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BACHELOR THESIS. BIOMEDICAL ENGINEERING

PREPARATION AND CHARACTERIZATION OF NEW ANTIBACTERIAL NANOCOMPOSITE MATERIALS BASED ON LDPE MATRIX

Author: Gloria María Pontes Quero

Tutor and director: Dania Olmos

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Abstract

Nowadays, bacterial biofilm contamination is one of the biggest problems in packaging materials. This research aims to prepare low-cost antimicrobial materials to be used in food industry, for example in food containers, to inhibit the growth of certain bacteria and prevent the spread of different diseases. One of the most popular synthetic materials used in food industry is polyethylene (PE) due to its low price, chemical resistance and good procesability. The antimicrobial activity of silver has been well-known for decades and used for several medical applications and as a disinfectant for many years. Using silver nanoparticles (AgNPs) instead of larger particles may increase the potential antibacterial effects of silver due to its high surface-to-volume ratio. Thereby, a nanocomposite of PE and AgNPs can be a promising low-cost and antimicrobial material for food industry. In particular, to perform these studies, low-density polyethylene (LDPE) was selected as bulk material and AgNPs as the filler.

LDPE-AgNPs composite materials were prepared with the aid of high-energy ball milling to obtain a homogeneous dispersion of the nanoparticles through the polymeric matrix. Then, powders obtained from the milling process were hot pressed to prepare films and study their final properties and in-service behavior. The effects of processing conditions as well as the presence of the nanoparticles in the final properties of the materials were analyzed by means of scanning electron microscopy (SEM) and contact angle (CA). SEM revealed a homogenous dispersion of the AgNPs in the polymer matrix although aggregates of the particles were still present. Contact angle measurements confirmed that surface properties of the materials were not modified due to the presence of silver particles. The behavior of the materials and their resilience to microbial growth was tested against a strain of *Escherichia Coli* (DH5α). Two studies were done to characterize the antibacterial character of the materials: i) Kirby-Bauer diffusion test and ii) bacterial cultures on the surface of the materials. Results showed that the presence of AgNPs inhibits to certain extent biofilm formation on the surface of the materials. In addition, image analysis of the size and aspect ratio of the bacteria was done. It was found that the aspect ratio of the bacteria changes from neat polyethylene to materials with silver particles.

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1. Introduction

Nowadays food preservation, quality maintenance, and safety are major growing concerns of the food industry. **Food industry** and **food safety** deal with the handling, preparation and storage of food. In this sense, research on storage of food products is a key factor to prevent food-borne infectious diseases. New food packaging technologies are developing as a response to consumer demands or industrial production trends towards mildly preserved, fresh, tasty and convenient food products with prolonged shelf-life and controlled quality. The ability of many bacteria like *Escherichia coli* to adhere to surfaces and to form biofilms has major implications in several industries including the food packaging industry. Biofilm communities provide several advantages to their members including easy access to food and nutrients and resistance to antibiotics, creating a persistent source of contamination (Van Houdt & Michiels, 2010). In Figure I.1, colorized scanning electron micrograph images of *E. Coli* and *Clostridium difficile* are shown as an example. *Clostridium difficile* is a major cause of antibiotic-associated diarrheal disease. In Figure I.2, biofilms of bacteria developed on a catheter (a) and on the surface of HDPE (b) are presented as an example of bacterial growth on different surfaces.

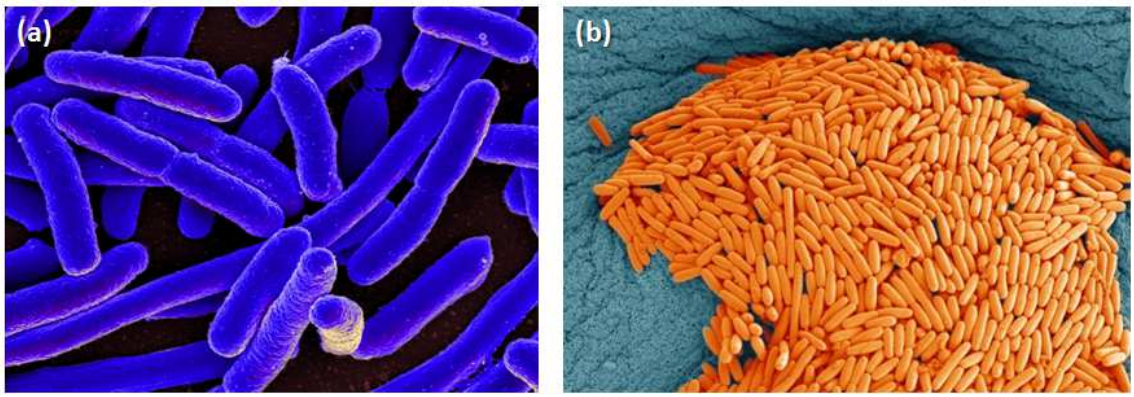

Figure I.1. (a) Colorized scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip (from NIAID); (b) *Clostridium difficile* colony (from David Goulding, Wellcome Trust Sanger Institute. Wellcome Images).

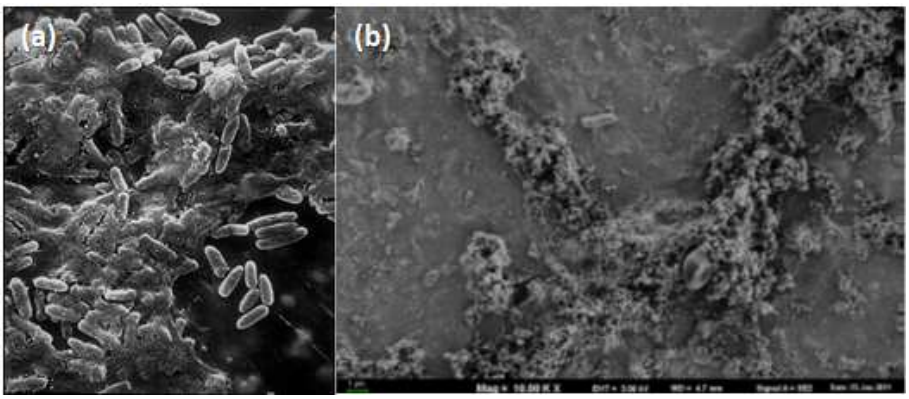

Figure I.2. (a) Biofilm on a catheter (Source: Wichita State University); (b) Bacterial biofilm on HDPE surface (Rozej *et al.*, 2015).

In this sense, packaging materials play an important role both in food and health sectors. *Controlled released packaging* and *antimicrobial packaging* are two alternatives of the so called **active packaging** widely used for this purpose. *Controlled release packaging (CRP)* is an emerging technology by which active compounds such as antimicrobials or antioxidants are first incorporated into the package and then released to the food in a controlled manner to inhibit microbial growth or other food deteriorations, thereby extending the shelf-life of the food product. *Antimicrobial packaging* is related to the incorporation of antimicrobial agents and is able to inhibit the growth of pathogenic microbes, thus increasing the shelf-life of foods and other products or substances in the packaging materials.

In the food industry, **polyethylene (PE)** is a popular polymer used in packaging for decades. Its chemical resistance to solvents, its versatility and low price, among other properties, make PE one of the most common polymers used in food packaging applications. LDPE stands out because it is semi-rigid, translucent, very tough, weatherproof, has good chemical resistance, low water absorption and easily processed by most methods (Vasile, 2016). However, PE has not antimicrobial effect itself and new active packaging solutions must be searched to bring together PE properties with the antimicrobial properties of other materials.

It is a well-known fact that **silver ions** and **silver-based compounds** are highly toxic to several microorganisms which include different species of bacteria. Materials in the nanoscale have a naturally enhancement of their properties due to the high surface-to-volume ratio occurring at this scale. Recent advances in nanotechnology have, therefore, opened the door to the use of silver as nanoparticles. These particles can be used as additives to produce nanocomposites of polymeric materials with antibacterial effect.

Polymer nanocomposites are quite widespread in many fields due to their countless applications. However, to attain their unique properties homogenous and uniform particle dispersions should be obtained. So far, many attempts were done to distribute particles uniformly within a polymer matrix. In general, when the ratio between the amount and size of particles is too high, uniform dispersion of them is rarely achieved. Recently, using high-energy ball milling (HEBM) it was possible to prepare materials with an efficient dispersion of nanoparticles within different thermoplastic polymers (Sorrentino *et al*., 2005; Castrillo *et al*., 2007).

Hence, taking into account the abovementioned, in this project antimicrobial polymer nanocomposites based in **low density polyethylene (LDPE)** and **silver nanoparticles (AgNPs)** will be prepared as a potential low-cost solution for biofilm prevention to prepare materials with potential applications in food industry.

2. Objectives

2.1. General objective

The aim of this work is to prepare plastics based on low-density polyethylene (**LDPE**) filled with silver nanoparticles (**AgNPs**), resistant to bacterial growth and biofilm development with potential applications in food industry.

2.2. Specific objectives

This general objective can be divided into four more specific objectives that would together achieve the overall goal of the project as follows:

a) **Preparation of materials with different amounts of AgNPs**. In particular, four concentrations were selected: 0%, 0.5%, 1% and 2% (%wt). To disperse the nanoparticles in the polymer matrix, high-energy ball milling (HEBM) will be used followed by a subsequent hot-pressing step to obtain films of the materials.

b) **Characterization of the dispersion of the particles** within the polymer matrix. For this purpose, high-resolution scanning electron microscopy (SEM) and X-ray microanalysis will be used.

c) **Characterization of the surface properties of the materials.** The effect of the presence of AgNPs on surface properties of the materials will be studied with the aid of contact angle measurements.

d) **Study of materials response against bacterial growth.** In this case, an *E. Coli* strain (DH5α) was selected for this purpose. Two different studies will be done:

i) Kirby-Bauer diffusion test, to study the efficacy of the materials against bacterial growth as a function of the distance to the surface of the material.

ii) Studies of bacterial growth and biofilm development on the surfaces of the materials. For this purpose, cultures of *E. Coli* on the surface of the materials will be done. SEM will be used to visualize changes in the amount of bacteria present on the surface of the materials. Also, image analysis will be done to study morphological changes, if any, in the bacteria induced by the presence of AgNPs.

3. Background

3.1. Plastics in the packaging industry

The term plastic usually refers to synthetic or semi-synthetic processable materials based on polymers. **Plastics** are typically organic polymers of high molecular mass, but they often contain other substances. They are usually synthetic, most commonly derived from petrochemicals, but many are partially natural. Nowadays, plastics can be shaped in many different ways to serve myriad purposes, from air bags to cell phones. The polymer and the additives can be fabricated to differ in the chemical composition and structure allowing developing tailor made products for a wide range of applications. This adaptability serves today's packaging needs and makes packaging one of the main fields of applications of plastic materials (Piringer & Baner, 2008). Some examples of polymers used in the packaging industry are: polypropylene, polystyrene, polycarbonate and polyethylene.

Plastics, in contrast to more traditional packaging materials like glass or metals, have some drawbacks as they are permeable to the exchange of low molecular weight compounds such as gases, they undergo scalping of packaged food constituents and are willing to migration into foodstuffs of packaging constituents (Baldevraj & Jagadish, 2011). In spite of this, the range of shapes in which plastics can be arranged, their ease of processing and handling, their low price and their excellent chemical resistance have made them very useful for packaging purposes.

Among the most important functions of packaging materials are food preservation as well as quality maintenance and safety. Usually, **food packaging systems** are designed to preserve food and delay the undesirable effects of external agents on food quality. This requires retardation of deterioration or decomposition of food, extension of shelf-life, and maintenance of quality and safety of the packed food. Packaging protects food from environmental factors such as heat, light, oxygen, pressure, enzymes, odors, microorganisms, insects, dirt and dust particles, gaseous emissions, and more. All of these factors may cause deterioration of foods and beverages. Packaging systems need to prolong shelf-life of food retarding enzymatic, microbial, and biochemical reactions by different approaches as can be temperature and moisture control; addition of chemicals such as antioxidants or other substances like salt, sugar, carbon dioxide, or natural acids or removal of oxygen (Brody *et al.* 2008).

Because almost no pure polymer exhibits all the desired mechanical and barrier properties required for every conceivable food packaging application, complex multilayer films, polymer blends or polymeric matrices filled with particles are often used. New active and intelligent packaging polymer based systems are gaining importance by offering numerous and innovative solutions for extending the shelf-life or maintain, improve or monitor food quality and safety. **Active packaging** refers to the incorporation of certain additives into packaging films trying to maintain and extend product shelf-life. In contrast to traditional packaging, intelligent packaging can change the composition and characteristics of food interacting with the product and the environment and playing a dynamic role in food (Dobrucka & Cierpiszewski, 2014).

Polymer nanocomposites are one of the latest materials used in food packaging. They are created by dispersing an inert, nanoscale filler in a polymeric matrix. Filler materials include clay and silicate nanoplatelets, silica nanoparticles, carbon nanotubes, graphene, starch nanocrystals, cellulose-based nanofibers or nanowhiskers, chitin or chitosan nanoparticles and inorganic particles. The most obvious application of polymer nanocomposites is to enhance polymer barrier properties but they are also stronger, more flame resistant and possess better thermal properties than polymers with no nanoscale filler (Duncan, 2014).

3.1.1. Polyethylene

Polyethylene (PE) is a synthetic polymer created through the polymerization of the gas ethylene. It is the most produced plastic and it is used primarily in the packaging industry but it has many other applications such as the production of shopping bags, molded housewares, children toys, containers or coatings. Ethylene is a hydrocarbon that has a simple chemical structure with formula C_2H_4. It is a stable molecule that polymerizes when in contact with a catalyst in a highly exothermic reaction (Piringer & Baner, 2008). It is particularly useful where moisture resistance and low cost are required. Its competitive nature makes PE the cheapest translucent plastic available.

PE consists of nonpolar, saturated, high molecular weight hydrocarbons. It is a thermoplastic, which softens when heated and hardens when cooled, and which can be heated and cooled repeatedly. It is available in a wide range of rigidities and other properties depending on the production process, where high density materials are the most rigid. This polymer has high permeability to oxygen and carbon dioxide and excellent resistance to alkaline substances, acids and solvents. It has good chemical resistance and good fatigue and wear resistance. The relatively good resistance of PE to sunlight and changes in humidity are other important features for most applications, especially for packaging (Kresser, 1957).

Some other important characteristics are the maintenance of its physical properties, its flexibility and its electrical insulating nature. It has good tracking resistance although it becomes easily electrostatically charged. The mechanical and thermal properties of PE are dependent on its structure, its molecular weight and its distribution, crystallinity and the type and amount of comonomer, temperature, and stress (Vasile, 2016). Its strength, rigidity and hardness are low but it is highly ductile and has low friction. It is semi-crystalline because of its symmetric molecular structure. It can be transparent, translucent and opaque depending on its thermal history and thickness of the film.

There are different types of PE depending on its density and branching and their properties vary between them. Among the main types of PE are **high-density polyethylene (HDPE), low-density polyethylene (LDPE)** and **linear low-density polyethylene (LLDPE**). HDPE chains are linear and obtained at relatively low temperatures and pressures. It is flexible but more rigid than LDPE. It is semi-translucent depending on density and has good impact strength, stress crack and chemical resistance. LDPE chains are branched and polymerized at very high pressures and temperatures. It is very flexible, translucent with high impact strength and good

chemical resistance. Finally, LLDPE is a substantially linear polymer with significant numbers of short branches (Billmeyer, 1984).

The **applications** of plastics in general and of PE in particular are innumerable. Its applications are based on the combination of one or several properties. The range of densities, formulations and styles for this plastic are substantial on their own, and with different uses for each variation, make the possibilities for this product incredibly broad. Moreover, the low cost of PE production has encouraged producers to prefer its use over many other plastics. LDPE is mainly used as a film, extrusion coating, injection moulding, wire and cable, adhesives and sealants, sheets and blow moulding (Vasile, 2016). LDPE is increasingly used in the medical field, the automotive sector, cosmetics and liquid packaging. HDPE is also used in pipes for canalization, injection moulded products, industrial containers, packaging, housewares, and many more applications.

PE is widely used for **food and drink packaging**. LDPE is the largest volume single polymer used in food packaging in either film or blow-molded form. The high degree of branching with long chains gives molten LDPE unique flow properties. LDPE is used for both rigid containers and plastic film applications such as plastic bags and film wrap. It can be easily blow-molded into bottles where its flexibility enables the contents to be squeezed out. HDPE has high tensile strength and it is blow-molded into bottles for different liquid packaging applications. While other applications are available, LLDPE is used predominantly in film applications due to its toughness, flexibility, and relative transparency (Robertson, 2006).

3.1.2. Composite materials

A **composite material** is made of two assembled materials of different natures that allow obtaining a new material in which the set of performance features is greater than that of the components separately. Usually, a composite is formed by one or more discontinuous phases spread out in a continuous phase. The discontinuous phase is normally harder and has superior mechanical characteristics and is called the *reinforcement* or *filler*, while the continuous phase is called the *matrix* (Berthelot, 1999). Although composites can be homogeneous, the individual elements do not change and confer the composite their distinct features without loss of identity or characteristics, as the different materials do not dissolve or blend into each other.

With composite materials we can make a better use of the advantages of some materials while minimizing their deficiencies. This optimization allows the designer to overcome constraints related to selection of convectional materials. In this way, the properties of the material can be tailored to satisfy particular design requirements. Some of the typical engineered composite materials are reinforced plastics, cements, concrete and metal or ceramic composites. There is a wide range of composite materials and the different types have different performance characteristics. They can be classified in different ways based on: its function, its preparation process, the type of reinforcing material, the type of matrix material or the geometry of the dispersed phase (Wang *et al*., 2011).

Plastics used for packaging applications such as HDPE or LDPE usually degrade during their lifetime without additives. Package materials are no longer single elements but rather are comprised of several different materials. The **additives** can be organic or inorganic chemicals enabling processing of plastics, shaping their use and enhancing their end-use performance (Piringer & Baner, 2008). The **reinforcement** material is thought to improve mechanical and barrier properties of polymers. For instance, when incorporated to polymer matrices, they may interact with the food or the surrounding environment, providing active properties to packaging systems. Such properties, when present in food packaging systems, are usually related either to improvements in food safety or to information about the safety status of a product (Azeredo *et al*., 2011).

3.1.3. Active materials

Innovations in the packaging industry have been limited to a small number of commodity materials such as barrier materials like new polymers, complex and multilayer materials, with new designs. However, food packaging has no longer just a passive role in protecting and marketing a product (Dobrucka & Cierpiszewski, 2014). The concept of **active or intelligent packaging** for food applications has been recently exploited, obtaining for the package an active role in the preservation, health-promoting capacity and provision of information concerning the products. This is particularly important in the area of fresh and extended shelf-life foods.

Nowadays, packaging is being designed to influence consumer health by integrating active materials in the packaging structure, through bioactive packaging strategies. Some of the latest strategies combine bioplastics and nanotechnology (Baldevraj & Jagadish, 2011). **Nanotechnology** has a great interesting potential in active packaging because nanostructures may enhance desired properties or may introduce new additional effective functionalities. **Active packaging** properties are based either on the intrinsic properties of the polymer used as packaging material or on the introduction of specific additives in the polymer (Dobrucka & Cierpiszewski, 2014). Besides, active packaging involves the incorporation of substances into packaging systems in order to maintain or extend product quality and shelf-life.

There are different types of active packaging intended to have different outcomes. Packaged food has a certain level of gases and oxygen. A high level of oxygen reacts with the product and accelerates its degradation and oxidation reducing its shelf-life and its nutritional value. Oxygen scavengers and antioxidant agents are two solutions to reduce and control the residual levels of oxygen inside the package. A complementary approach to oxygen scavenging is the impregnation of the packaging with CO_2 since it suppresses microbial activity and therefore prolongs shelf-life of packed food. However, CO_2 changes the taste of some products (Prasad & Kochhar, 2014).

Control of ethylene plays a key role in packaging of fruits and vegetables. Most vegetables release ethylene after being harvested and ethylene accelerates ripening and causes vegetable deterioration. Some of the solutions to control ethylene are the use of polymers as LDPE and HDPE able to absorb ethylene or the use of ethylene scavengers. Furthermore, addition of essences and odors in the food packaging can increase the desirability of the product to the

consumer (Prasad & Kochhar, 2014). Another example of active materials is **antimicrobial materials.** In order to prevent microbial contamination of foods some packaging materials incorporate antimicrobial substances in or coated onto the packaging materials (Dainelli, 2008). In particular, in this work, we will focus on the development of antimicrobial materials incorporating active particles into the polymer matrix to test their resistance against biofilm development and bacterial growth.

3.2. Antimicrobial particles

The adhesion and proliferation of bacteria on material surfaces is highly related to the appearance of human infection, including the posterior formation of antibiotic-resistant biofilms in both healthcare and industrial applications. Thereby, antibacterial agents are very important in health and food industry sectors to prevent the development of biofilms and bacterial growth. Before chemotherapeutics appeared, inorganic antimicrobials such as silver and copper were used since ancient times to treat microbial infections. Nowadays, the development of nanotechnology has allowed us to use nanosized inorganic and organic particles as antimicrobial agents (Palza, 2015).

Antimicrobial particles have different activities on diverse pathogenic microorganisms due to their various physiologies. Antimicrobial particles are integrated either directly into food or to the packaging material where it is released over a period of time to maintain the product quality, as well as its safety leading to its extended shelf-life. Characterization of microorganisms may be very helpful in order to choose the antimicrobial agent. Antimicrobial packaging in films prevents microbial growth on the food surface by direct contact of the packaging material with the surface of food (Kharkwal *et al.,* 2015). New polymer and composite materials with antimicrobial properties are one of the most promising active packaging systems for food packaging industry.

Nanoparticles have unique physical and chemical properties which can be suitably manipulated for specific applications. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. The nanosize and the large surface area of the nanoparticles enhance their interaction with microbes and allow them to carry out a wide range of antimicrobial activities. Inorganic based nanoparticles constitute an effective antimicrobial agent against pathogenic microorganisms. Therefore, some metal nanoparticles like silver, titanium dioxide and zinc oxide are receiving considerable attention as antimicrobial additives in consumer, health-related and industrial products (Robertson, 2006).

3.2.1. Silver nanoparticles

For centuries, people have used silver for antibacterial applications in the medical field such as for burn treatments. Silver is a natural, non-toxic, hypoallergenic element that does not accumulate in the body to cause harm. Due to the large surface area relative to volume that appears at the nanoscale, nanoparticles usually have enhanced properties compared to the

bulk material of the same element. Regarding silver nanoparticles, the antimicrobial effect is enhanced and low amounts of nanoparticles can provoke antibacterial effects to hundreds of square meters in the host material (Theivasanthi & Alagar, 2011).

Silver nanoparticles have been studied as a medium for antibiotic delivery, and to synthesize composites to use them as disinfecting filters and coating materials. The mechanism by which silver nanoparticles cause antimicrobial effects is not clearly known and is under discussion. The high affinity of silver to sulfur and phosphorus is one of the main facts that may provoke the antimicrobial effect. The bacterial cell membrane has a high number of sulfur containing proteins allowing the reaction of silver with sulfur-containing amino acids in the cell membrane, affecting bacterial viability (Rai & Bai, 2011).

The attachment of the nanoparticles to the surface of the cell membrane disrupts permeability and respiration functions of the cell. It is also proposed that silver nanoparticles not only interact with the surface of a membrane but can also penetrate inside the bacteria (Rai & Bai, 2011). The formation of free radicals by the nanoparticles can be considered as another way of inducing cell death since these free radicals are able to damage the cell membrane creating pores that lead to the death of the cell since the bacterial cell wall is essential for the survival of the organism (Dobrucka & Cierpiszewski, 2014).

3.3. Nanoparticles and nanocomposite materials

3.3.1. General characteristics of nanocomposites

Nanotechnology is science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers. This scale is the one at which quantum effects dominate the properties of materials, much biology occurs and surfaces and interfaces play a large role in material properties and interactions. The properties of materials change as their size approaches the nanoscale. Bulk materials possess mostly constant physical properties independently of their size. However, as materials reach nanosizes the percentage of atoms at the surface increases compared to the total number of atoms of the material bulk. This can lead to changes of nanoparticle properties which are partly due to the fact that surface dominates over the bulk properties (Rai & Bai, 2011).

Therefore, nanoscale structures such as nanoparticles have very high surface-to-volume and aspect ratios making them ideal to be used in polymeric materials (Prabhu & Poulose, 2012). As surface area per mass of a material increases, a greater amount of the material can come into contact with surrounding materials, thus affecting some properties of materials. In general, the fact that most biological processes occur at the nanoscale enables scientists to use the unique physical, chemical, mechanical, and optical properties of materials that naturally occur at that scale (Vasile, 2016). By creating nanometer-scale structures we can control important material properties such as the melting temperature, magnetic properties or the charge capacity without modifying the chemical composition of the material.

A **nanocomposite** is a multiphase solid material where one of the phases has one, two or three dimensions of less than 100 nanometers or structures having nanoscale repeat distances between the different phases that make up the material (Prabhu & Poulose, 2012). Polymer nanocomposites are formed by a polymer (thermoplastic, thermoset or elastomer) and a supporting nanoscale material (nanoparticle). These nanocomposites have significant improvements in certain properties such as mechanical properties, thermal stability or gas barrier properties. There are several factors that affect the characteristics of polymer nanocomposites: the morphology of the polymer nanocomposite, the type of nanoparticles used and their surface treatments or the features of the polymeric matrix (Koo, 2010).

Nanocomposites, where the nano-sized reinforcement (nanoparticles) is uniformly dispersed through the polymeric matrix, have very large interfacial area per volume and the distances between polymers and fillers are very short. Polymer nanocomposites exhibit multifunctional, high-performance polymer characteristics beyond what traditional filled polymeric materials have. Some of the multifunctional characteristics that polymer nanocomposites possess are the improvement of thermal resistance and flame resistance, moisture resistance, lower gas permeability, charge dissipation and chemical resistance (Koo, 2010).

Antimicrobial nanocomposites include nanosized antimicrobial particles. Because of the high surface-area-to-volume ratio of nanosized antimicrobials, these systems can inhibit more microorganisms when compared to higher scale counterparts. Exposure to nanoparticles present in food packaging can occur through dermal contact, inhalation, or ingestion of nanoparticles which have migrated to food. Nanoparticles may eventually be released into the environment and enter the food chain indirectly (Azeredo *et al*., 2011). Therefore, toxicological aspects of the nano-antimicrobial materials have also to be taken into account.

3.3.2. Nanocomposites of polyethylene with silver nanoparticles

One of the most popular synthetic materials used in food industry is polyethylene due to its low price, chemical inertness, good electrical properties and relatively easy processing. However, an increasing concern due to bacterial growth in polymeric surfaces has led to the idea of producing composite materials which have incorporated antibacterial agents in their compositions. Polymer-nanoparticle materials are present within the composites, with the nanoparticles having the role of antibacterial agents. Different types of antibacterial particles such as Cu, ZnO, TiO_2, MgO, and Ag have been inserted into polymer films to create antibacterial materials.

In most cases of fresh or processed food products, microbial contamination occurs on the surface of the food due to the post-process handling. Therefore, it is necessary to effectively control microbial growth on the surface of the food using antimicrobial active packaging films. It has been reported that materials with silver nanoparticles are one of the most effective antimicrobial materials due to silver's strong toxicity against various types of microorganisms (Azeredo, 2013). Therefore, nanocomposites based on **polyethylene** and **silver nanoparticles** seem to be a promising material for applications in which bacterial inhibition is needed, such as **medical** and **packaging applications**.

The antibacterial effect of nanocomposites has been attributed to the release of ions from the nanoparticles. Bacterial viability studies revealed that the antimicrobial effect of the nanocomposites is dependent on the amount of nanoparticles. While most of the work attributed the antibacterial action of the nanocomposites to the release of silver ions from silver nanoparticles, it is demonstrated that the penetration of nanoparticles has also a role in the antibacterial ability of the nanocomposite (Azlin-hasim *et al.*, 2016). It has been shown low migration of silver from the nanocomposite films into food products. Moreover, it has also been reported that the exposure results fell well below than the exposure limits for all extreme conditions of the scenarios tested, suggesting silver-LDPE nanocomposites are suitable to be in contact with non-acidic foods because they are less likely to facilitate migration (Tamayo *et al.*, 2014).

3.4. Dispersion of nanoparticles

3.4.1. High-energy ball milling (HEBM)

In the last years, there has been an increasing interest in organic polymer-inorganic particles nanocomposites, since they exhibit unique properties which will depend on the particles used to fill the polymer. Normally, these enhanced properties can be achieved if there is an adequate dispersion of the nanoparticles within the polymer matrix. It is generally stated that physical mixtures of organic polymers with inorganic particles lead to separation in discrete phases with agglomeration of particles resulting in poor mechanical and optical properties. For this reason, different methods have been tried to achieve a good dispersion of nanoparticles into polymer matrices. However, most of them required long processing times, use of solvents, chemical modification of the matrix and/or the filler, and even sometimes high processing temperatures (Olmos *et al*., 2012). Nevertheless, they do not seem to ensure uniform dispersion in terms of isolated nanoparticles when sizes of the particles are less than 50 nm and filler loads are greater than 5% by weight (wt). **High-energy blending by ball milling** might be an interesting alternative not only because of its potential results but also from an economical point of view (Castrillo *et al.*, 2007).

High-energy ball milling (HEBM) is a ball milling process where a powder mixture placed in the ball mill is subjected to high-energy collision from the balls. A ball mill consists of a hollow cylindrical shell rotating about its axis. The axis of the shell may be horizontal or at a small angle to the horizontal. It is partially filled with balls. The grinding media is the balls, which may be made of steel, stainless steel or ceramic. The inner surface of the cylindrical shell is usually lined with an abrasion-resistant material such as manganese steel or rubber. Ball milling has several advantages over other systems. The cost of installation and grinding medium is low, it is suitable for both batch and continuous operation, similarly it is suitable for open as well as closed circuit grinding and is applicable for materials of all degrees of hardness (Takacs, 2002).

Recently, using HEBM, homogeneous dispersion of nanoparticles in thermoplastic matrices has been achieved (Olmos *et al*., 2012). Usually, HEBM provides good results with small, chemical modifications, if any, of the polymer or the nanoparticles, avoiding, at the same time, the use of solvents and high processing temperatures. For example, in Figure 1 (a), is shown as an example a scanning electron microscopy image to show the dispersion of $BaTiO_3$ nanoparticles in polystyrene (PS) matrix. In Figure 1 (b) an atomic force microscopy image obtained with the phase contrast mode is shown to illustrate the dispersion of isolated TiO_2 nanoparticles in HDPE.

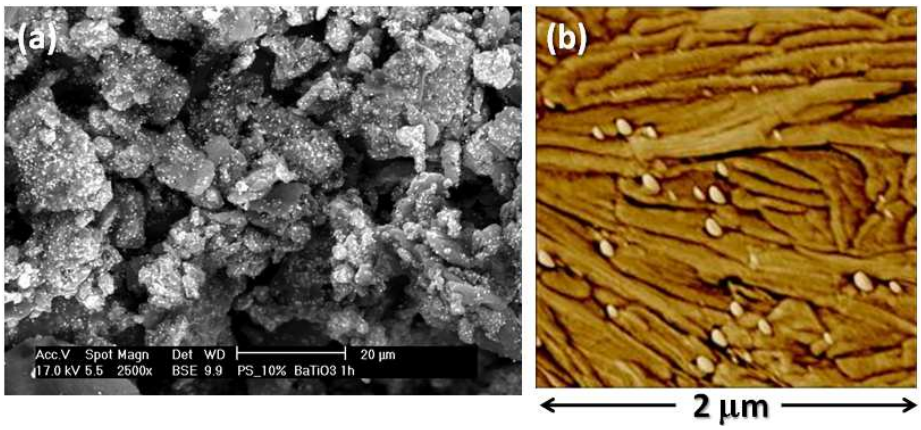

Figure 1. (a) Scanning electron microscopy image obtained with the backscattered detector of the milled mixture of PS with 10% (wt%) of $BaTiO_3$ particles (reproduced from Olmos *et al.*, 2013); (b) atomic force microscopy image (phase contrast) showing isolated 65 nm sized TiO_2 nanoparticles dispersed in HDPE (Olmos *et al*., 2009).

3.5. Harmful bacteria in food

Microbial growth in packaged food significantly decreases its safety and the security of public health. Most food is perishable and food spoilage is caused both biologically and chemically. Most spoilage processes are due to biological mechanisms like auto-degradation by enzymes or microbial, viral, protozoa and parasite contamination. Microorganisms are the major route for food spoilage which cause a decrease in the quality product, shorten its shelf-life and can induce some pathogenic problems. Microbial spoilage of food can be caused by different types of yeasts, mold and bacteria and their growth depends on many factors such as temperature or pH. Therefore, it is difficult to prevent food spoilage and different microorganisms may require different types of growth prevention (Cushen *et al.*, 2014).

Foodborne illness is any disease due to food spoilage of contaminated food, harmful bacteria, viruses, parasites or natural toxins like poisonous mushrooms. Foodborne illness arises from improper handling, preparation, or food storage and one of the most common responsible are pathogenic bacteria. Some of the usual bacteria involved in this type of illness are *Escherichia coli, Salmonella, Listeria, Cryptosporidium*, *Campylobacter*, *Clostridium botulinum, Clostridium perfringens, Shigella, Vibrio parahaemolyticus* and *Vibrio vulnificus* (Ebnesajjad, 2013).

Specific bacteria are associated with a certain environment or food. For instance, *E. coli* is a common indicator of fecal contamination while *Vibrio* species are associated with the consumption of fish or shellfish. While some bacteria are found in several environmental conditions, others only live in specific food such as *C. botulinum* which plagues canned products (Barros-Velázquez, 2016). Other example is *Listeria monocytogenes* which potentially contaminates ready-to-eat foods such as smoked meat and sausages.

Although the food environment is a leading factor of bacterial specification, the outbreaks can also be promoted by living environment such as seen with Shiga toxins from *Shigella*. With Shiga, the disease is caused by unsanitary conditions after natural or human disasters. The people affected and the degree of illness also vary among bacteria (Caya, 2001). While most of them such as *Salmonella, Campylobacter* and *E. coli* are likely to cause illness in young or immunosuppressed people, other bacteria focus on a specific group of people like *L. monocytogenes* affecting primarily to pregnant women (Barros-Velázquez, 2016).

Most bacteria are associated with natural or abiotic surfaces and grow as biofilm rather than as planktonic cells which are floating as a single cell in water. A biofilm is a microbial derived community characterized by cells attached to a living surface and embedded in a matrix of extracellular polymeric substances or EPS that they have produced. This polymicrobic group has an altered phenotype and it is physiologically different from planktonic microorganisms (Gómez *et al.*, 2014). Bacteria growing in a biofilm are highly resistant to biocide and antibiotic agents so new effective methods for the prevention of biofilm formation are needed for both, medical and food packaging applications.

Although traditional food preservation methods such as drying, heating, freezing, fermentation and salting can extend food shelf-life, they do not inhibit the growth of pathogenic microorganisms. Antimicrobial packaging is a new development that incorporates antimicrobial agents into polymer matrix to suppress the activities of different microorganisms (Mari & Vrane, 2007). Antimicrobial packaging is a form of active packaging which interacts with the product to eliminate or to prevent the growth of bacteria. The target microorganisms and the food composition must be considered in antimicrobial packaging. As with any antimicrobial, those to be incorporated into polymers have to be selected based on their spectrum of activity, mode of action, chemical composition, and the rate of growth and physiological state of the targeted microorganisms (Sung *et al*., 2013).

3.6. Scanning electron microscopy as tool for the study of biofilm development

In scanning electron microscopy (SEM), a fine probe of high-energy electrons (with energies typically up to 40 keV) is focused on a specimen, and scanned along a pattern of parallel lines. The signals derived from electron-sample interactions reveal information about the sample including external morphology, chemical composition (using X-Ray microanalysis), crystalline structure and orientation of the materials making up the sample. Various signals are generated as a result of the impact of the incident electrons, which are collected to form an image or to

analyze the sample surface. These are mainly secondary electrons, with energies of a few tens of eV, high-energy electrons backscattered from the primary beam and characteristic X-rays (Bogner *et al.*, 2007).

Various imaging techniques have been employed in the investigation of bacterial biofilms. SEM is a useful technique for the investigation of surface structure of biological samples and much of the current knowledge about biofilms is due to the advances in SEM imaging studies (Duckett & Ligrone, 1995). For example, electron-microscopic studies proved that biofilms are composed of bacteria wrapped in a dense exopolysaccharide matrix (Blenkinsopp & Costerton, 1991). SEM plays a paramount role for assessing the microbial populations, three-dimensional structure, physiology, thickness, etc. SEM proved to be an invaluable method for ultra-structural investigation, allowing imaging of the overall appearance and/or specific features of biofilms formed in different environments, e.g. microbial colonies and individual cells, the glycocalyx, and the presence of inorganic products within the biofilm.

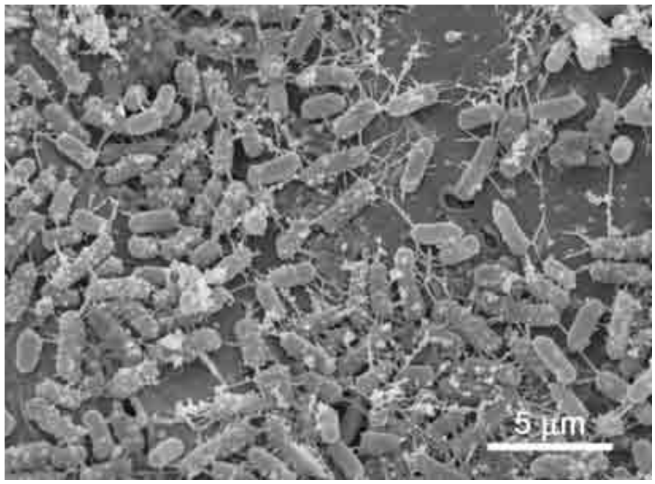

Figure 2. SEM image showing attachment and biofilm formation by *E. coli* cells in an apple. Taken from Annous *et al.,* 2009.

Among the advantages of SEM is the higher resolution for visualization of microbial biofilms than other imaging techniques. Nevertheless, this technique uses graded solvents (alcohol, acetone, and xylene) to gradually dehydrate the specimen prior to microscopy visualization, since water is not compatible with the vacuum used with the electron beam (Donlan *et al.*, 2002). While any pretreatment can alter specimen morphology, drying appears to significantly alter biofilms due to EPS polymers collapsing (Fassel & Edmiston, 1999).

In conclusion, SEM offers researchers a powerful research tool and a reliable method to study in detail the structure of biofilms. However, since it requires high-vacuum conditions, the wet materials and biological samples must undergo a complex preparation that limits the application of SEM on this kind of specimen and often causes the introduction of artifacts. Thereby, an improvement in the technology can be expected, in order to derive the maximum benefit from the microscopic study of biological tissue.

4. Experimental work

4.1. Materials

The materials used for the preparation of the films were: LDPE in the form of pellets (melt index 25.00 g/10 min, 190 ºC/2.16 kg, ASTM D 1238, density = 0.93 g/cm^3, melting point 116 ºC) supplied by Sigma Aldrich and silver nanoparticles (average diameter 50 nm, spherical) supplied by HWNANO Materials.

All solvents were HPLC quality and were used without further purification.

4.2. Sample preparation

4.2.1 High-energy ball milling (HEBM)

In order to facilitate the milling process with the silver nanoparticles, PE pellets were firstly grinded in a miller MF 10 Basic. The grinding process was carried out at 1500 rpm obtaining the polymer in the form of flakes. This grinded PE was further used as a control sample. Once the PE flakes were formed, high-energy ball milling was used to disperse the AgNPs in the PE matrix. The process was done with a commercial mixer Retsch MM400. The samples were introduced in two stainless steel vessels of 50 ml each with fifteen milling balls of 9 mm diameter. The filling level of the vessel is limited by the following settings: one third of the total volume is occupied by the sample whilst other third is occupied by the balls. The remaining third is the free vessel volume, essential for the powder and the ball motion during agitation.

Four samples were prepared: PE (control) and PE filled with 0.5%, 1% and 2% of AgNPs (mass percent). The milling process was done immersing the vessels filled with the samples and the milling balls in liquid nitrogen for 15 minutes. Next, the vessels were placed in the MM400 mixer milling machine and subjected to one milling cycle for 5 minutes using a vibration frequency of 25 kHz. This cycle was repeated 12 times to complete 1 hour of active milling. Previous results (Serra *et al*., 2012) suggest that crosscontamination of the samples milled under cryogenic conditions were less than 0.03% (wt/wt) as determined by atomic absorption. Grinded PE was also studied as reference material to assess the effect of milling, if any, in the final properties of PE.

Powders with different concentrations of AgNPs are presented in Figure 3. As can be seen, powders containing no silver have a whitish color while powders containing AgNPs are silver in color. Bigger flakes that appeared in grinded PE were not present after the milling process. A relative homogeneous dispersion of nanoparticles was achieved after the milling process when observing the powders.

Figure 3. Physical appearance of the different nanocomposites after the grinding and milling processes.

4.2.2. Films preparation

The powders obtained from the milling process were then subjected to hot pressing to obtain homogenous films of the different materials. The films were prepared using a press (FONTUNE PRESSES) by placing the milled powders between two square sheets of polyimide (Kapton®) of 12x12 cm^2. To control the thickness of the film, a mask of the same material was used with a window of 10x10 cm^2 where the powders were placed. The whole assembly was covered altogether with two metallic plates (see Figure 4). Five films were prepared: grinded PE, milled PE (PE subjected to high-energy ball milling) and PE filled with 0.5%, 1% and 2% of Ag nanoparticles.

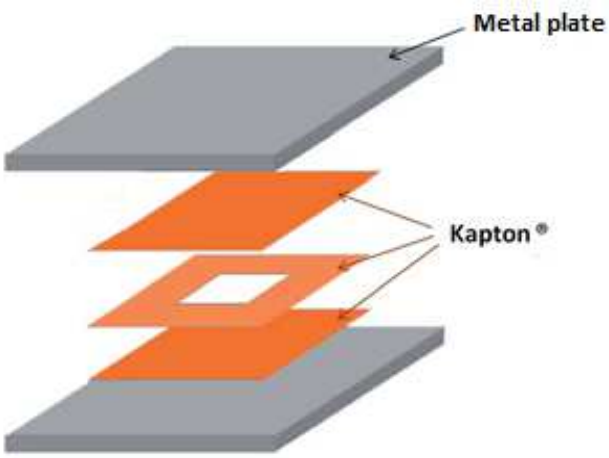

Figure 4. Experimental set-up used for film processing.

To prepare the films, 1.5 g of the different powders obtained after the milling process were deposited inside the mold. This system was placed on the press using the pressure-temperature cycle presented in Figure 5. Temperature was raised from ambient temperature to 140 ºC and maintained constant for 40 minutes. After this period, it is cooled down again to room temperature. Pressure is applied until 20 kN is achieved. It is maintained constant for 5 minutes and increased again until 70 kN is achieved, value maintained for 40 minutes. Pressure is then decreased following the same scheme used to increase its value. The films were cut in four pieces and re-press at the same conditions to obtain more homogeneous films.

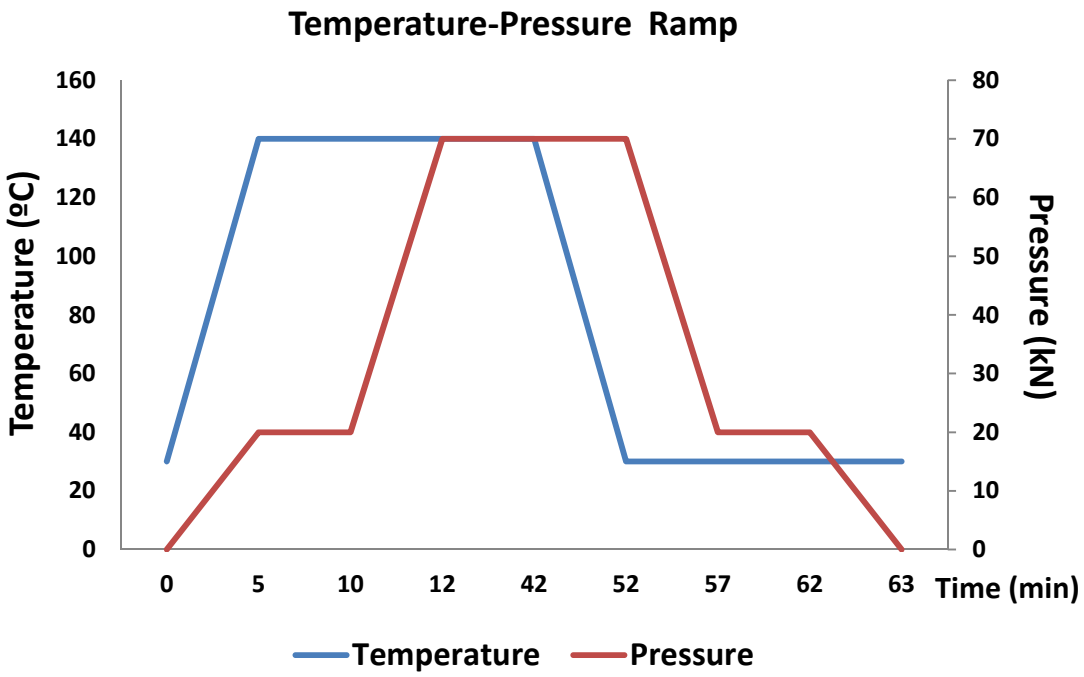

Figure 5. Temperature-pressure ramp.

Nanocomposite films were obtained in squares of 10x10 cm^2 after hot pressing (see Figure 6). As can be seen, films corresponding to grinded and milled PE are transparent while films containing increasing concentrations of AgNPs have a brownish color that increases in intensity as nanoparticles wt% increases. The films with nanoparticles present some type of gradients visible to the naked eye. These gradients may indicate the presence of higher concentrations of nanoparticles in the darkest areas of the film, thus suggesting that total dispersion of the particles was not fully achieved.

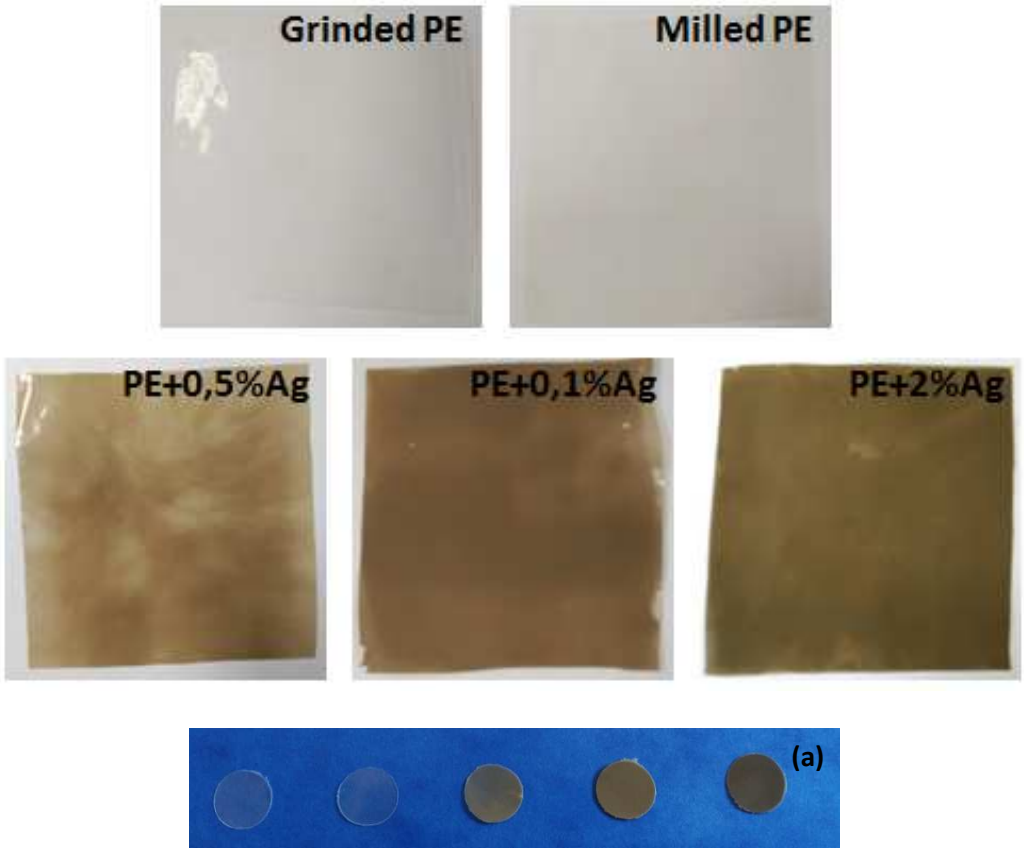

Figure 6. Physical appearance of nanocomposite films obtained after hot pressing. (a) shows the same films after die cutting for better visualization.

4.3. Materials characterization

4.3.1. Scanning electron microscopy (SEM)

To assess the dispersion of the nanoparticles within the polymer matrix a scanning electron microscope FEI's Teneo SEM was used. This microscope provides ultrahigh resolution enabling us to visualize the nanoparticles. The films were placed on copper double sided sticky tape and then gold coated. In all cases, samples were placed on the sample holders with double sided conductive sticky tape and then subjected to sputtering with gold for 45 seconds making them conductive and avoiding 'charging artifacts' during SEM examination. Micrographs of sample surfaces corresponding to PE + 0.5% Ag, PE + 1% Ag and PE + 2% Ag were taken to visualize AgNPs on these surfaces. Energy-dispersive X-ray spectroscopy (also known as EDS microanalysis) was also performed to confirm the presence of the nanoparticle within the film. The micrographs were collected using a voltage of 10.0 kV and a filament current of 0.2 nA. The working distance was set at 10 mm.

On the other hand, to evaluate the growth of bacteria and biofilm development on the surface of the materials after the cultures, a SEM Philips XL30 was used. The samples were first fixed with glutaraldehyde and then dehydrated with ethanol prior to their visualization. Then, the samples were fixed with sticky tape on the SEM sample holder and gold coated. Micrographs with different magnifications were used to be able to study different characteristics of these cultures. More specifically, micrographs at different magnifications (50x, 1,000x, 2,500x and 6,500x) were collected. The voltage was set at 10 kV and working distance at 10 mm.

4.3.2. Contact angle (CA)

In general, the formation of bacterial biofilms takes place over three stages: a reversible adsorption step, primary adhesion of microorganisms to a surface, and colonization. Hydrophilic uncharged surfaces showed the greatest resistance to protein adsorption as shown by Cunliffe *et al*., 1999. Then, hydrophilic surfaces can be an option to prevent cell adsorption to synthetic substrates making bacteria unable to attach and develop in biofilms. Since material surface properties have been reported to significantly affect bacterial attachment and biofilm development, contact angle measurements and free surface energy calculations were carried out.

The contact angle is the angle, conventionally measured through the liquid, where a liquid interface meets a solid surface. It quantifies the hydrophobicity of a solid surface by a liquid via the Young equation. A high contact angle indicates a low solid surface energy or chemical affinity. A low contact angle indicates a high solid surface energy or chemical affinity, and a high degree of wetting (see Figure 7). One of the parameters characterizing the surfaces of materials is the surface free energy that quantifies the disruption of intermolecular bonds that occur when a surface is created.

The most common way to determine its value is to measure the surface tension by the sessile drop method. The method consists of placing a droplet of liquid with a known surface energy on the surface of the solid to be studied. The shape of the drop, specifically the contact angle, and the known surface energy of the liquid are the parameters which can be used to calculate the surface energy of the solid sample.

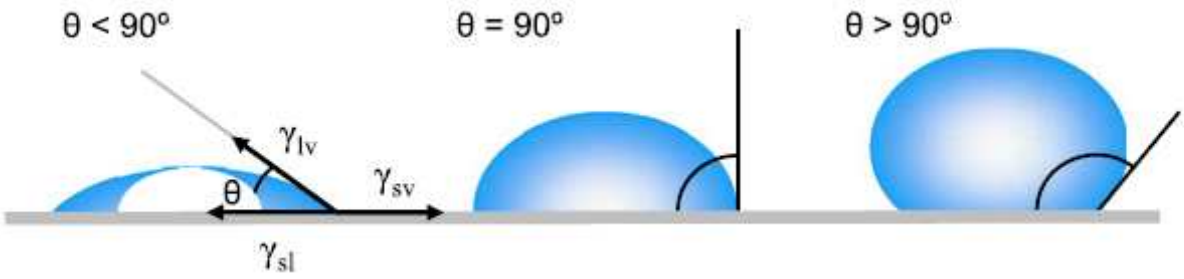

Figure 7. Illustration of contact angles formed by sessile liquid drops on a smooth homogeneous solid surface showing: a low contact angle (left); a 90º contact angle (center) and a high contact angle (right). (Taken from Yuan & Lee, 2013).

The Oss and Good acid-base method is a method used to calculate the surface free energy of a solid from the contact angle with three liquids. In doing so, the surface free energy is divided into a dispersive surface energy and subdivides the polar component as being the sum of two more specific components: the surface energy due to acidic interactions (σ^+) and due to basic interactions (σ^-). The acid component describes the propensity of a surface to have polar interactions with another surface that has the ability to act basic being an electron donor. The base component of the surface energy describes the propensity of a surface to have polar interactions with a second surface that acts acidic being an electron acceptor (Janczuk *et al*., 1993). The principle equation for this theory is:

$$\sigma_L (\cos\theta + 1) = 2 [\sqrt{\sigma^D_L}\, \sigma^D_s + \sqrt{\sigma^-_L}\, \sigma^+_s + \sqrt{\sigma^+_L}\, \sigma^-_s] \qquad (1)$$

(where L and S stand for liquid and solid respectively, D means dispersive and θ is the contact angle)

The best way to deal with this theory is to use at least three liquids: one with only a dispersive component to its surface energy, one with only a dispersive and an acidic or basic component, and either a liquid with a dispersive and a basic or acidic component or a liquid with the three components (Good & van Oss, 1992).

In this experiment, contact angles of the five samples were measured to assess the hydrophilicity and surface energy of the material. Measurements were performed using a contact angle measuring instrument (OCA 15 supplied by KRÜSS GmbH). Three liquids were used: water (dispersive, acidic and basic components), glycerol (dispersive and basic components) and diiodomethane (only dispersive component). 20 droplet measurements were done for each of the three liquids and each of the 5 samples. Reported data were obtained by averaging the results of these 20 measurements. Free surface energy for each material was calculated with the equipment software using the Oss acid-base method.

4.4. Bacterial cultures

4.4.1. Kirby-Bauer diffusion test

In order to study the possible diffusion of particles through the material and its surroundings, a modification of the traditional Kirby-Bauer test was performed. Kirby-Bauer test is a test which uses antibiotic-impregnated wafers to test if bacteria are affected by antibiotics. Wafers containing antibiotics are placed on an agar plate where bacteria have been cultured, and the plate is incubated. If the antibiotic has antibacterial effect there will be an area around the wafer where bacteria have not grown enough to be visible. This interface is called inhibition zone. The size of this zone depends on how effective the substance is at stopping the growth of the bacteria (Biemer, 1973).

In our project, this test was used to determine if AgNPs diffuse through the agar plate and whether higher concentrations of nanoparticles provoke and increasing diffusion and antibacterial effect as a function of the distance of the surface of the material.

To perform the test, Gram negative *E. coli* (DH5α™ Competent Cells) supplied by ThermoFischer Scientific were used to determine the bactericidal effect of the films. Bacteria were thawed for 5 minutes in ice. A dilution of 90 µl of thawed bacteria and 910 µl LB was prepared and left to incubate at 37 ºC for 30 minutes. From this solution, 200 µl were placed in a LB-agar plate. After homogeneous dispersion, films (cut in squares of 1x1 cm^2) were placed and arranged as shown in Figure 8. The culture was incubated at 37ºC overnight.

The analysis of the Kirby-Bauer test was carried out by imaging the samples with an Olympus optical microscope and measuring the distance of the inhibition zone formed in each sample. The images were captured using the appropriate image analysis software (analySIS getIT).

Figure 8. Agar plate with the films and the culture media used for Kirby-Bauer testing.

4.4.2. Study of biofilm development and bacterial growth on the surface of the materials

The same *E. coli* strain used for Kirby-Bauer diffusion test was used to determine the antibacterial effect on the surface of the films. Bacteria were stored frozen at -80 ºC. An aliquot of bacteria was thawed in ice. A mixture of 90 µl of thawed bacteria and 2910 µl of LB (Luria-Bertani culture medium) was prepared and incubated at 37 ºC overnight. Next day, a dilution of 1/100 of bacteria and medium was prepared. Films for visualization with SEM microscopy were prepared. Cultures for biofilm formation in the nanocomposite films were

performed in a 24-microwell plate (ThermoFischer Scientific). For their preparation, squared samples of the films were cut (0.8x0.8 cm^2) and fixed with an epoxy adhesive (92 NURAL, Henkel) onto SEM sample plates (10 mm diameter). Five samples were prepared in this way, one for each film: grinded PE, milled PE, PE + 0.5% Ag, PE + 1% Ag and PE + 2% Ag.

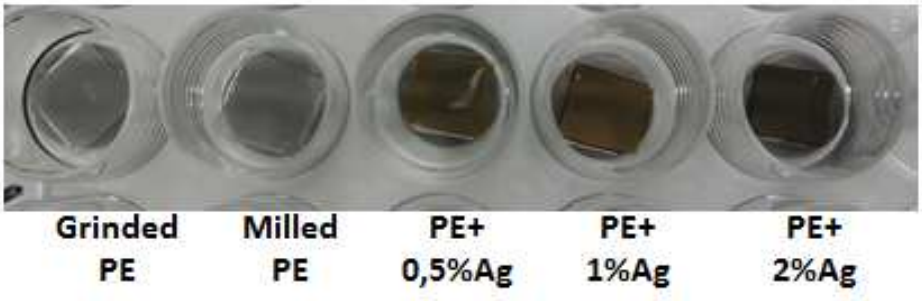

Figure 9. Disposition of the samples in the 24-microwell plate.

All the samples and, prior to incubation, were sterilized by spraying on a 70% solution of ethanol and posteriorly dry in a sterile laminar flow hood. From now on, all the processes were carried out in a sterile environment. Once the films were sterilized, 1 ml of the 1/100 dilution previously prepared was added to each well containing the samples and incubated for 3 hours at 37 ºC. After incubation, LB medium with loosely attached bacteria was removed, leaving only bacteria attached as a biofilm onto the surface of the materials. The films were gently rinsed with 1 ml of saline solution (NaCl 0.9 wt%) to remove excess of bacteria.

The first step in order to prepare the samples for SEM visualization is fixation. 1 ml of glutaraldehyde 2.5 wt% was added to each well and left for 30 minutes at room temperature to fix and kill bacteria onto the materials. After 30 minutes, glutaraldehyde was removed and samples were rinsed 3 times with PBS to remove remaining glutaraldehyde. After fixation, samples were dehydrated in four 10-minutes steps of increasing (30, 50, 70 and 100%) ethanol concentrations. Finally, ethanol was removed and samples were left in the laminar flow hood in order for them to dry completely.

5. Budget

The estimation of the total costs of the project results from costs of equipment (Table 1), materials (Table 2), culture assay materials (Table 3) and personnel involved during the experimental work (Table 4).

	Price/Hour	Nº of hours	Total price (€)
MF 10 Basic miller	10€/hour	25 hours	**250€**
Mixer milling Retsch MM400	10€/hour	18 hours	**180€**
Hot press Fontijne Presses TPB374	10€/hour	10 hours	**100€**
SEM (Philips XL30 and FEI's Teneo)	20€/hour	6 hours	**120€**
Olympus optical microscope	10€/hour	2 hours	**20€**
Contact angle equipment OCA 15 supplied by KRÜSS GmbH	10€/hour	15 hours	**150€**
			TOTAL: 820€

Table 1. Equipment costs.

	Price/Unit	Nº of units	Total price (€)
LDPE (Sigma Aldrich)	29,20€/jar 250g/jar 0.1168€/g 2.20g/film 0.26€/film	5 films	**1.3€**
Ag nanoparticles (HWNANO Materials)	230€/100g	0.1 g	**0.23€**
Kapton film	5 sheet/batch 69,42€/batch 13,88€/sheet	2 sheets	**27.76€**
Liquid Nitrogen	5€/liter	50 liters	**250€**
			TOTAL: 279.29€

Table 2. Material costs.

	Price/Unit	Nº of units	Total price (€)
24-microwell plate	1.13€/microplate	1	**1.13€**
Agar plate	66€/500g 0.13€/g 0.75g/plate	1	**0.1€**
Other lab material expenses (pipettes, LB medium, glutaraldehyde etc)	_____	_____	**3€**
			TOTAL: 4.23€

Table 3. Culture assay costs.

	Price/Hour	Nº of hours	Total price (€)
Biomedical engineer	15€/hour	**350 hours**	**5,250€**
Project collaborator	30€/hour	**70 hours**	**2,100€**
Project collaborator	30€/hour	**35 hours**	**1,050€**
Bioengineering laboratory technician	10€/hour	**10 hours**	**100€**
SEM technician	10€/hour	**2 hours**	**20€**
			TOTAL: 8,520€

Table 4. Personnel costs.

	Costs
Equipment	**820€**
Materials	**279€**
Culture Assay	**4€**
Personnel	**8,520 €**
	TOTAL COST: 9,623€

Table 5. Total cost of the project.

Summing the costs of personnel, the costs of the materials and equipment employed, the total costs of the project is **9,623 €**. In general, the materials used in the project were not very expensive. The higher costs are mainly due to labor costs.

6. Results and discussion

6.1. Dispersion analysis of nanoparticles and EDS microanalysis

SEM micrographs in Figure 10 correspond to the film surfaces of the composite samples under study before exposure to bacterial cultures. The images were collected using the backscattered detector. This detector is used because the intensity of the back-scattered electrons (BSE signal) is strongly related to the atomic number (Z) of the specimen. Figures 10 (a), (b) and (c) correspond to each of the composite samples with increasing silver content 0.5%, 1% and 2% of AgNPs, whereas Figure 10 (d) corresponds to a magnification done for sample PE + 1% Ag. Irrespective of the sample we can observe a dark matrix with bright spots that may be assigned to silver rich domains. All the samples show a relative uniform dispersion of AgNPs (represented by the brighter spots indicative of the presence of heavier elements such as Ag). It demonstrates that high-energy ball milling is a valid method to produce a homogenous dispersion of particles in a polymeric matrix.

The increasing content of silver is visible from the lowest to highest concentration (see Figure 10 (a)-(c)). There are some areas of variable size and shapes where particle aggregates seem to be present. However, these aggregates with an average dimeter of 400-500 nm (formed approximately by 10 nanoparticles each in diameter) were already present in the raw nanoparticles (see arrows in Figure 10 (a)). The formation of these aggregates may be promoted also by the re-press process during film preparation.

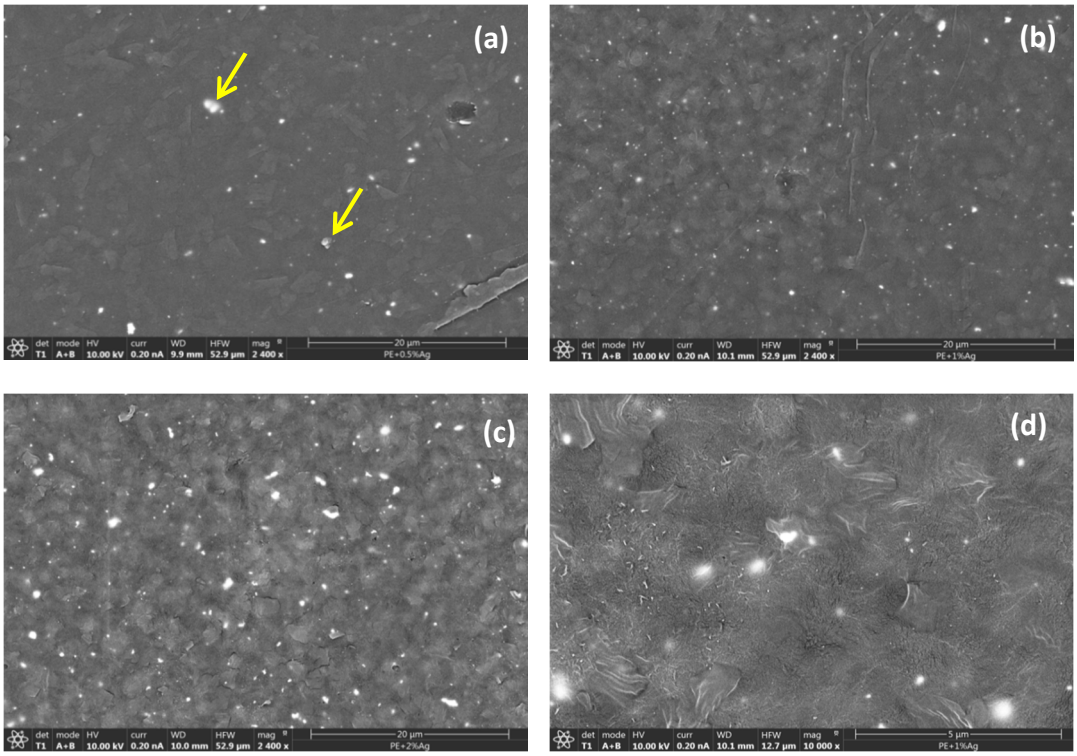

Figure 10. SEM micrographs with 2,400x magnification of the film surfaces of (a) PE + 0.5% Ag, (b) PE + 1% Ag, (c) PE + 2% Ag and (d) PE + 1% Ag with 10,000x magnification.

X-ray microanalysis of the PE + 2% Ag sample was carried out to verify the presence of silver nanoparticles in the sample. Spots 1 and 2 shown in Figure 11 (a) were selected for the study. The results confirmed that the bright points were indeed AgNPs embedded in the PE matrix as indicated by the microanalysis. This study also revealed that the samples contained carbon, the main element of polymer backbones, and gold, corresponding to the sputtering done during sample preparation for SEM. No signs of iron from the milling tools were detected indicating that contamination of the milling process in not present in the samples or if present, is below the detection limit of the system.

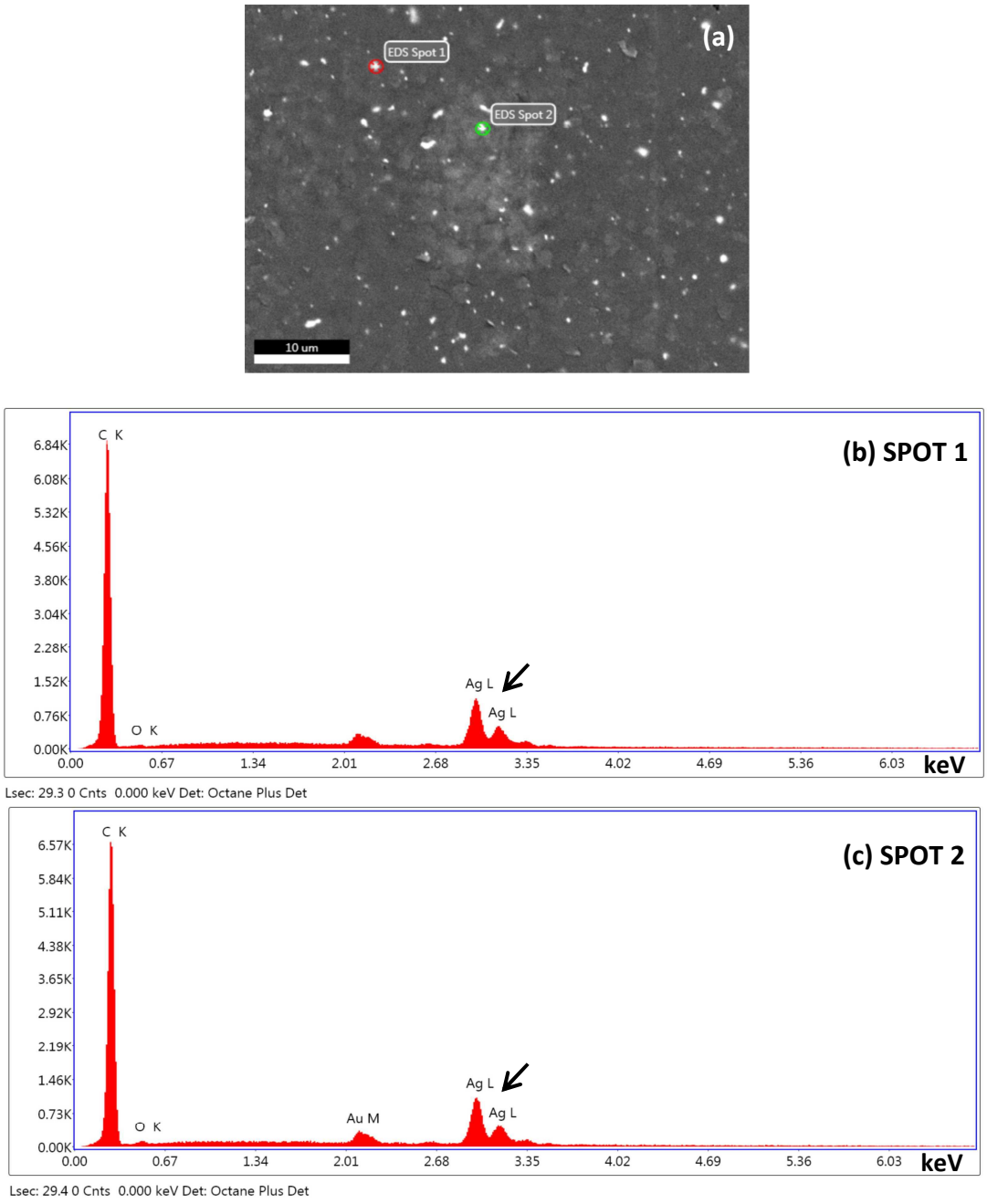

Figure 11. (a) SEM micrograph with the selected spots 1 and 2 for X-ray microanalysis. Results of X-ray microanalysis for (b) spot 1 and (c) spot 2.

6.2. Characterization of surface properties of materials

Figure 12 shows optical micrographs showing the profiles formed by water drops on the surfaces of grinded PE (a), milled PE (b), PE + 0.5% Ag (c), PE + 1% Ag (d) and PE + 2% Ag (e). These profiles were then used to determine the corresponding contact angles. The average values of the contact angles obtained in each case were also included. Results for mean contact angles for the materials under study for each of the selected solvents are collected in Table 6.

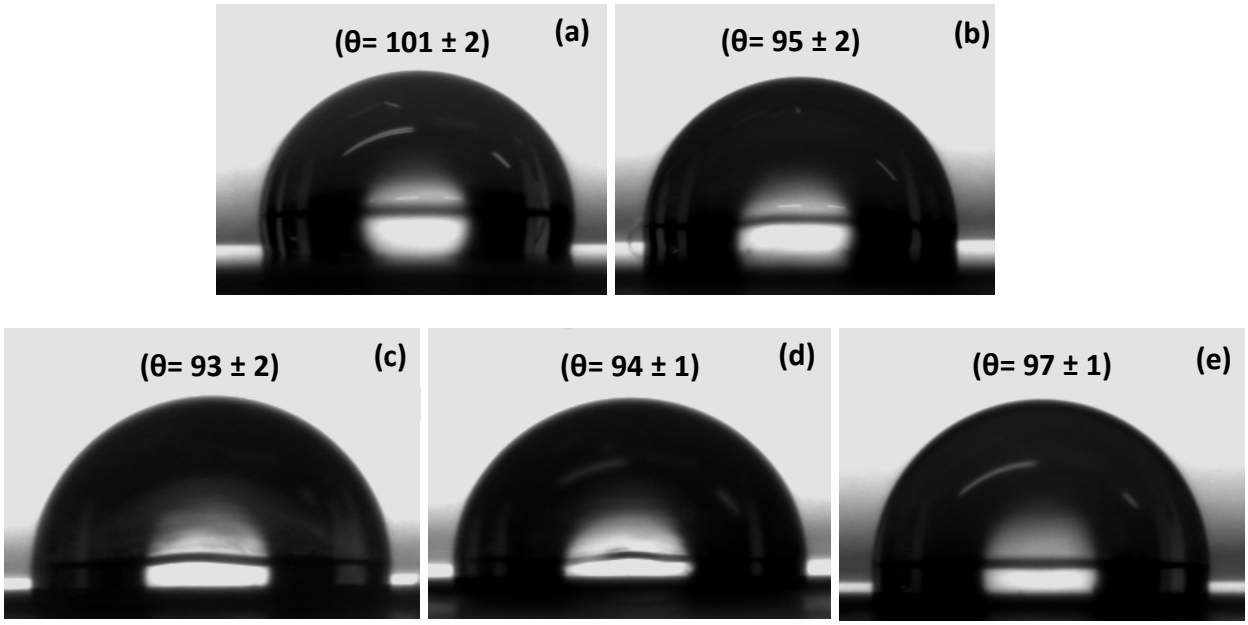

Figure 12. Micrographs showing water drop profile on (a) grinded PE, (b) milled PE, (c) PE + 0.5% Ag, (d) PE + 1% Ag and (e) PE + 2% Ag.

	Θ in water (º)	Θ in glycerol (º)	Θ in diiodomethane (º)
Grinded PE	101 ± 2	95 ± 2	74 ± 1
Milled PE	95 ± 2	94 ± 2	73 ± 2
PE + 0.5% Ag	93 ± 2	96 ± 1	72 ± 2
PE + 1 % Ag	94 ± 1	93 ± 2	73 ± 2
PE + 2 % Ag	97 ± 1	95 ± 1	72 ± 2

Table 6. Mean contact angles.

Small contact angles (<90°) correspond to more hydrophilic surfaces while large contact angles (>90°) correspond to hydrophobic surfaces. From our results, we can see that all the samples are hydrophobic since the mean contact angle for each is higher than 90º. Taking into account the standard deviation (used as a variability indicator), all films have roughly the same hydrophobicity with the exception of grinded PE which is slightly more hydrophobic than the rest. This may be due to the milling process, though the differences are not very high. However, the presence of AgNPs does not seem to affect the hydrophobicity of the sample surfaces since there are no significant changes in contact angle measurements.

Surface energies according to the van Oss method were calculated with the aid of the software. Results of the surface energy of the materials and its main components are presented in Table 7.

	Surface energy (mN/m)	Dispersive component (mN/m)	Acidic component (mN/m)	Basic component (mN/m)
Grinded PE	21 ± 2	21 ± 1	0 ± 0	3 ± 2
Milled PE	20 ± 2	20 ± 1	0 ± 0	5 ± 2
PE + 0.5% Ag	18 ± 2	18 ± 2	0 ± 0	7 ± 2
PE + 1 % Ag	21 ± 2	21 ± 1	0 ± 0	6 ± 2
PE + 2 % Ag	21 ± 2	21 ± 1	0 ± 0	4 ± 1

Table 7. Surface energy values (mN/m) and its main components (dispersive, acidic and basic, also in mN/m) as calculated with the van Oss method.

Regarding surface free energies, we can reach the same conclusions since the values are more or less the same if standard deviation is taken into account. Table 7 shows surface free energy for each sample and the main components of this surface energy. In the five samples, no acidic component has been recorded and a small basic contribution in all the cases. This result is consistent with the samples studied since PE consists of nonpolar hydrocarbons. The main contribution is due to dispersive surface energy because the main interactions between polymer chains are due to dispersion forces. Hence, the introduction of AgNPs does not affect significantly surface properties of the samples at least up to 2% of AgNPs.

6.3. Study of antimicrobial properties

6.3.1. Kirby-Bauer diffusion test

Optical micrographs were collected at different magnifications: i) 1x to obtain a general picture of the sample; ii) 2.5x to measure inhibition distances along all the perimeter of the samples and iii) 4x to show with more detail changes in the inhibition distances, if any. In Figure 13, images showing the general profile obtained for all the samples under study (at 1x) are shown. Also a detail of the sample PE + 2% Ag is shown in last image to illustrate how inhibition distances (marked in yellow) were calculated. The sample corresponding to PE + 2% Ag was accidentally burned during bacterial culture with the burner. The measurement of the inhibition distance was performed on the 2.5x magnification images. Around 40 measurements of the interface were done for each material.

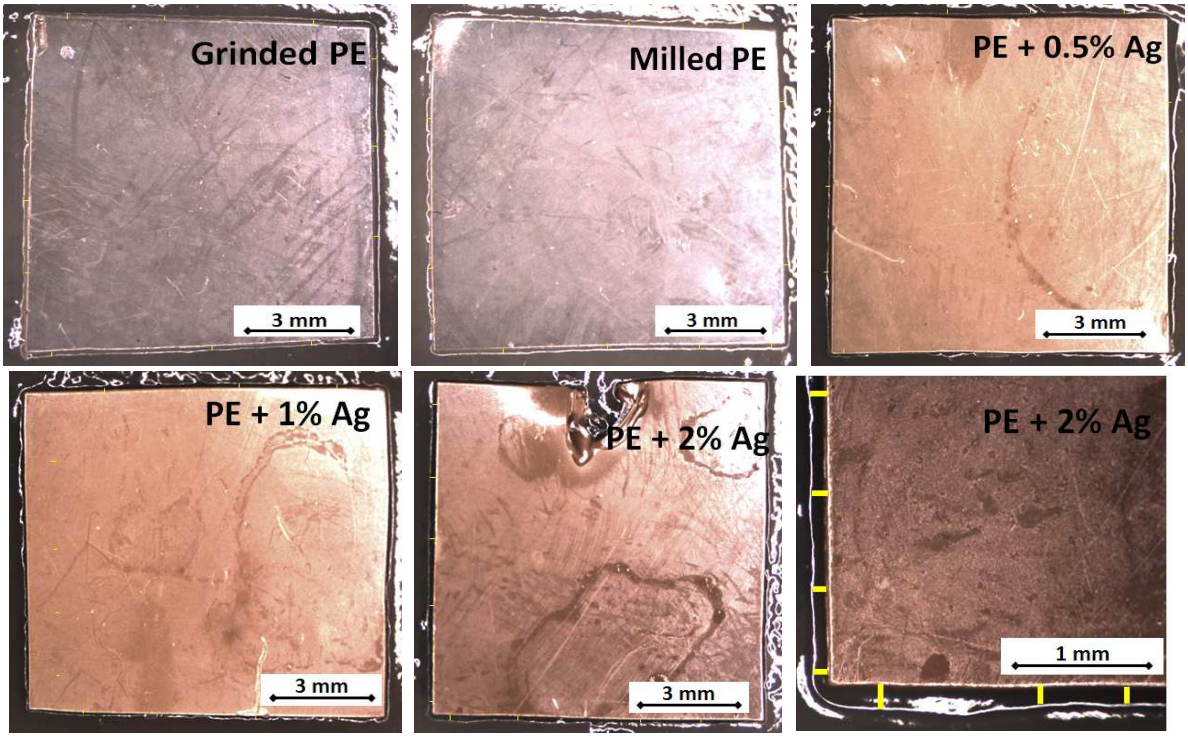

Figure 13. Images obtained with optical microscopy. First five images with a magnification of 1x. Last image corresponds to PE + 2% Ag with a magnification of 4x.

Results for the mean inhibition distances (in µm) calculated for each of the materials are presented in Figure 14. The error bars correspond to the standard deviation of the measurements which, in this case, was used to measure the variability of the distances. In this graph, it is shown that there is not an increasing interface distance as concentration of AgNPs is increased in the films. These interface distance is maintained around 100-120 µm from

materials with no silver content (grinded and milled PE) to materials with an increasing Ag wt%.

This test revealed that the presence of silver or silver ions in the material does not exert any effect out of the limits of the material itself. This means that the antimicrobial action of the particles (if any) will take place only on the surface or at very close distances of the surface of the material. This result is consistent with the samples under study. If we take into account that AgNPs are embeded in the material and the probability of diffusion of silver or silver ions into the agar medium is not very high due to the intrinsic viscosity of both the agar and the solid materials. Therefore, to test the efficiency of these materials against bacterial growth further studies of biofilm development on the surface of the materials were done.

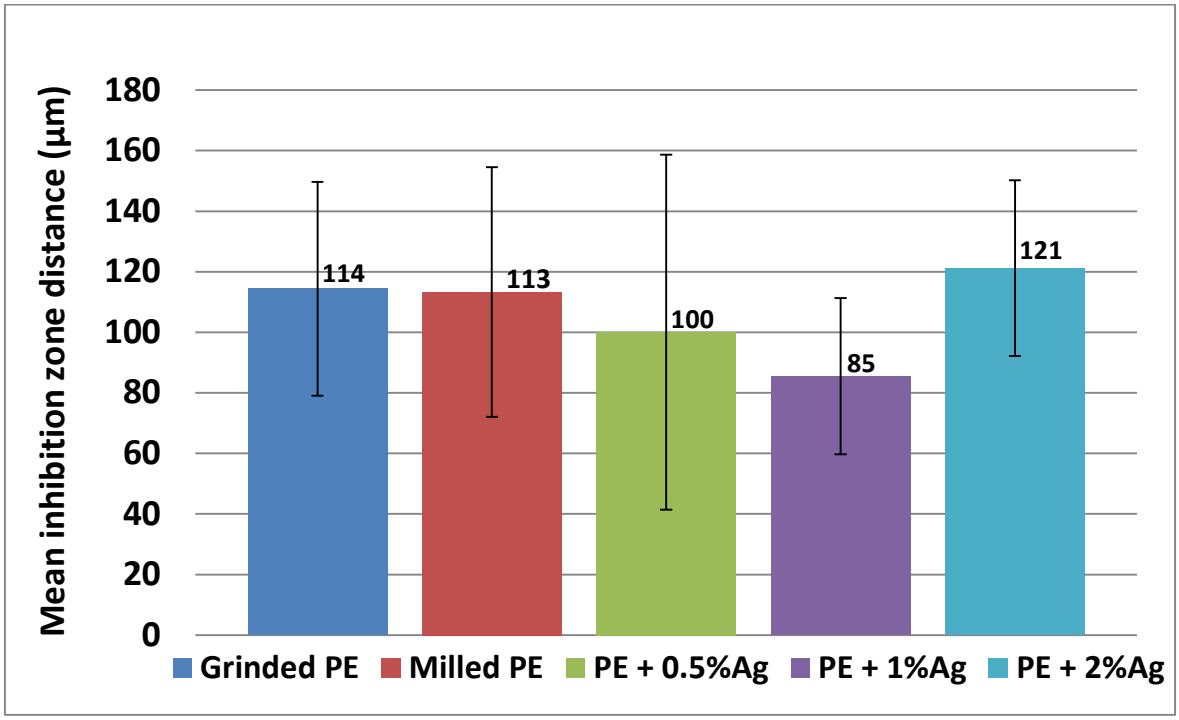

Figure 14. Mean inhibition zone distances (in µm) calculated for each of the materials under study. The standard deviation was used as a measure of the variability.

6.3.2. Study of biofilm development and bacterial growth on the surface of the materials

Figure 15 shows SEM micrographs (with 50x magnification) of the materials after doing bacterial cultures on their surface. From these images we can see that bacteria are arranged in two main zones, one with an oval form (marked with arrows in Figure 15 (a), (b) and (c)) and the other with groups of bacteria more dispersed among them. Initial observations may indicate that grinded and milled PE have more density of bacteria on their surfaces than

samples with AgNPs. Sample corresponding to PE + 2% Ag looks smoother than the rest which may mean a decreased concentration of bacteria on this surface.

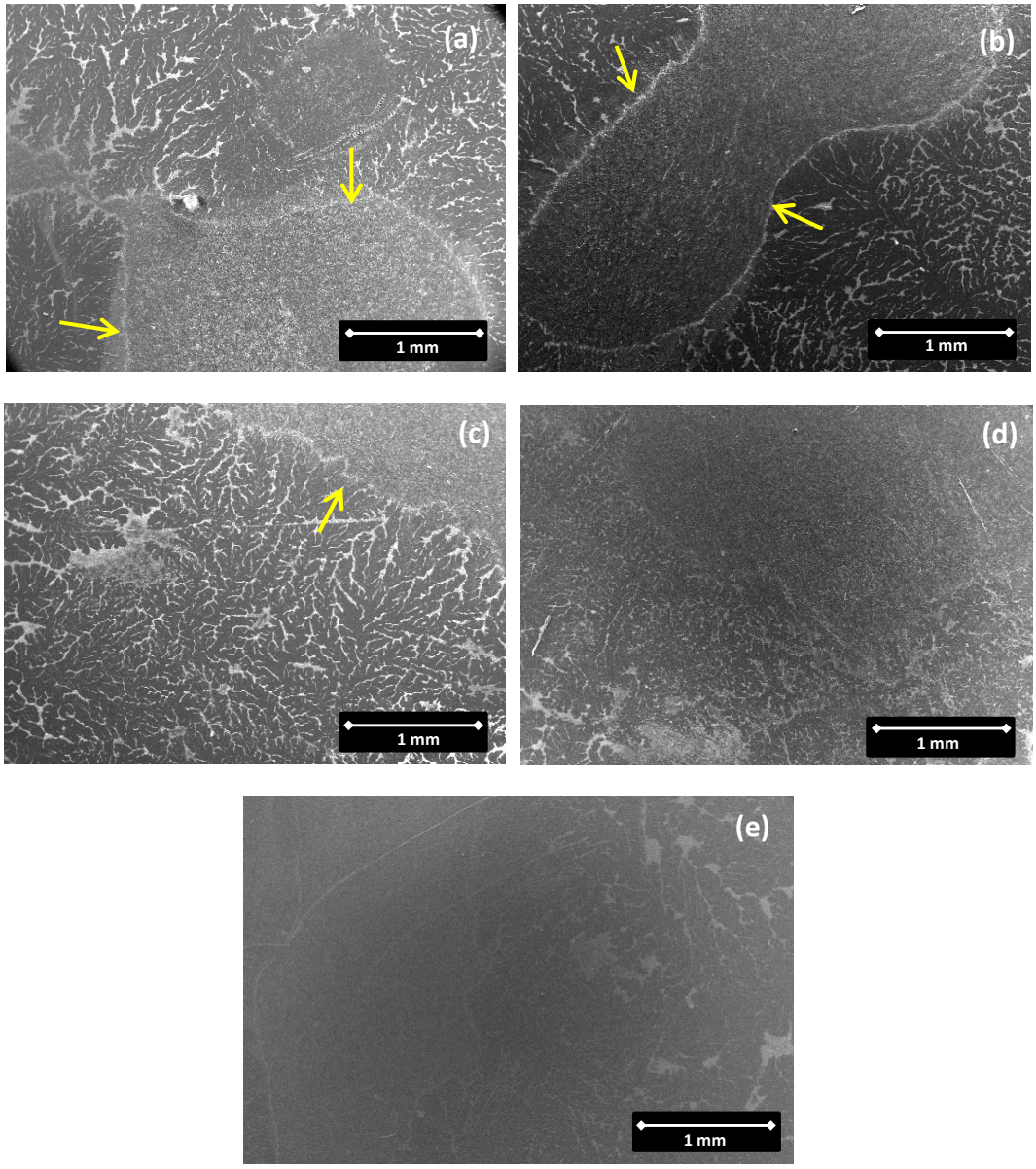

Figure 15. SEM micrographs with 50x magnification of the surfaces of the materials after doing bacterial cultures on (a) grinded PE, (b) milled PE, (c) PE + 0.5% Ag, (d) PE + 1% Ag and (e) PE + 2% Ag.

In order to analyse these observations with more detail, images at higher magnification were done. Figure 16 displays SEM micrographs obtained with 1,000x magnification for the same samples under study. The micrograph in Figure 16 (b) shows the boundary between the two well defined regions previously seen. As it is visible, the oval part has higher density of bacteria. In this oval region, bacteria seem to be closer to each other while in the other region there are some small aggregates of bacteria. It is also visible that there is a slightly decrease in

bacteria density as AgNPs increases in the films in materials corresponding to PE + 1% and 2% Ag. This effect is clearly seen when micrographs shown in Figure 16 (a) and (b), corresponding to PE are compared to those in Figure 16 (d) and (e), corresponding to PE + 1% and PE + 2% respectively.

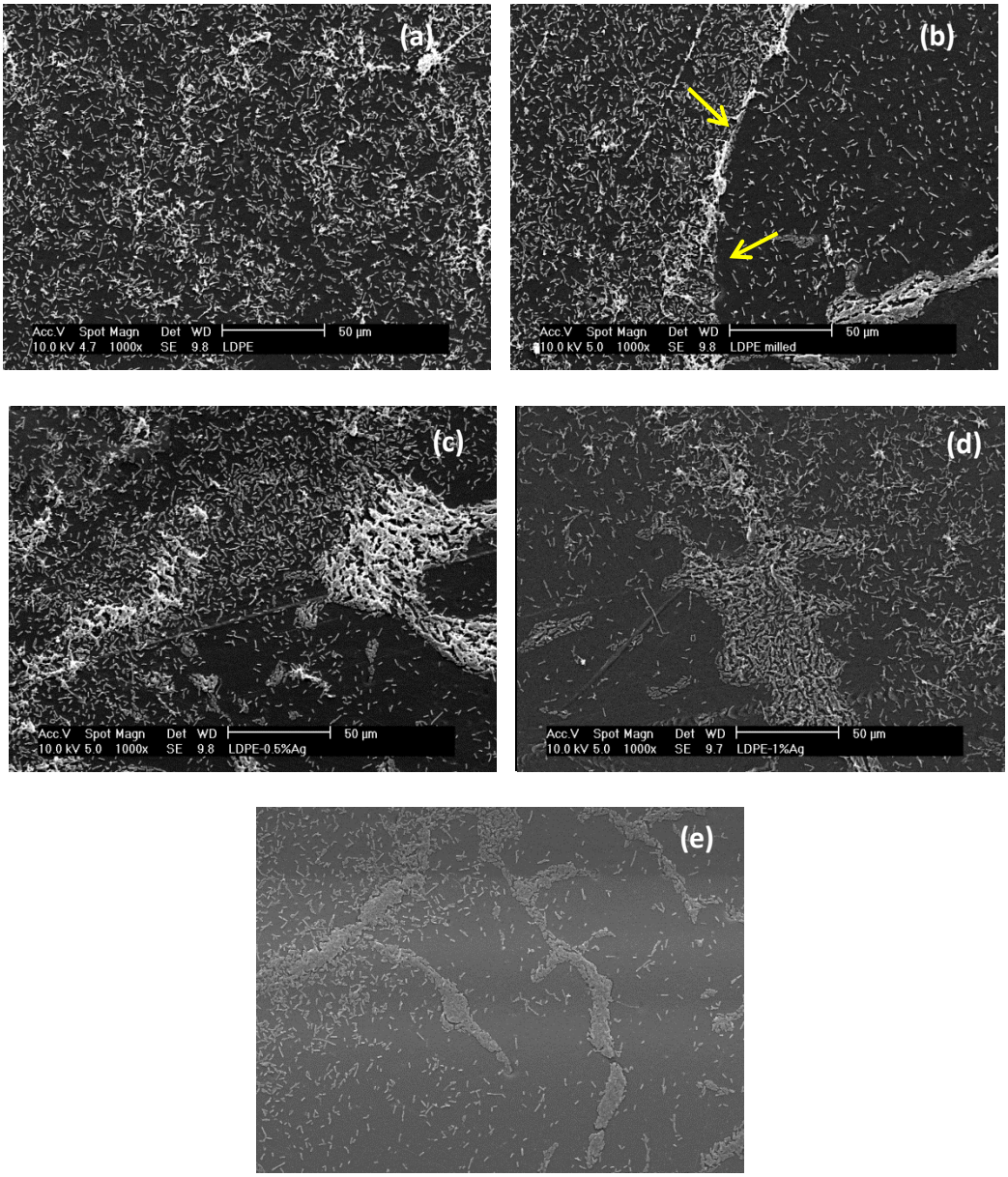

Figure 16. SEM micrographs with 1,000x magnification of bacterial cultures on (a) grinded PE, (b) milled PE, (c) PE + 0.5% Ag, (d) PE + 1% Ag and (e) PE + 2% Ag.

Images in Figure 17 correspond to the samples with 2,500x magnification. In this images, we can appreciate that bacteria in PE + 2% Ag are less dense. Therefore, it seems that the presence of AgNPs induces a decrease in the amount of bacteria since more space is visible

among them. It is also visible the boundary and the two regions described before in Figure 17 (b).

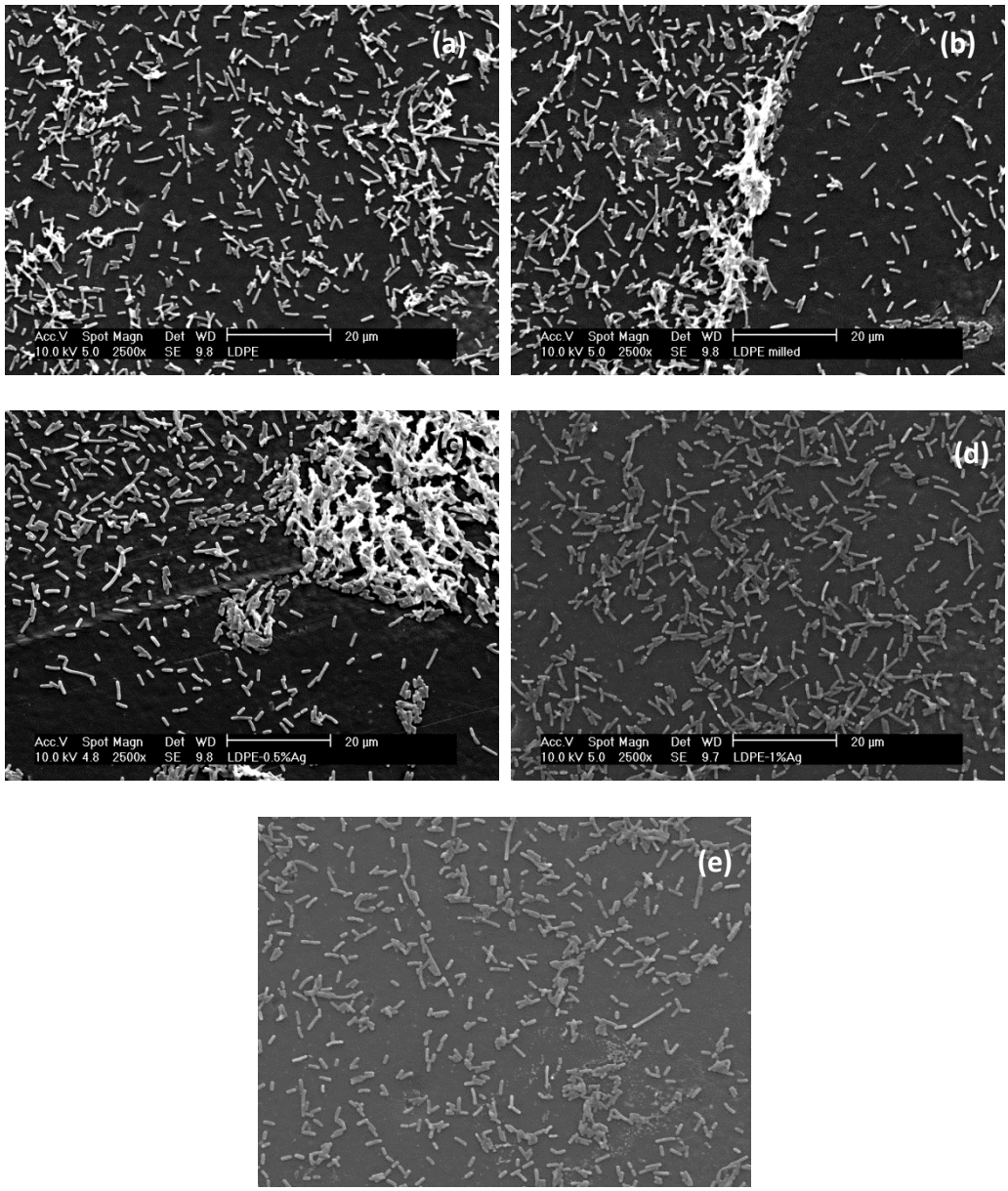

Figure 17. SEM micrographs with 2,500x magnification of the surfaces of the materials after doing bacterial cultures on (a) grinded PE, (b) milled PE, (c) PE + 0.5% Ag, (d) PE + 1% Ag and (e) PE + 2% Ag.

Finally, images in Figure 18 show that bacteria grew in the five samples although density seems to be reduced for the PE + 2% Ag film. Images captured some bacterial division (see arrow in Figure 18 (b)) and some linear aggregates (see arrow in Figure 18 (a)). Figure 18 (d) shows how bacteria arranged in the densely packed aggregates shown in previous figures. Bacteria grown

in PE with no silver content seem to be narrower compared, for example, with bacteria on PE + 2% Ag sample.

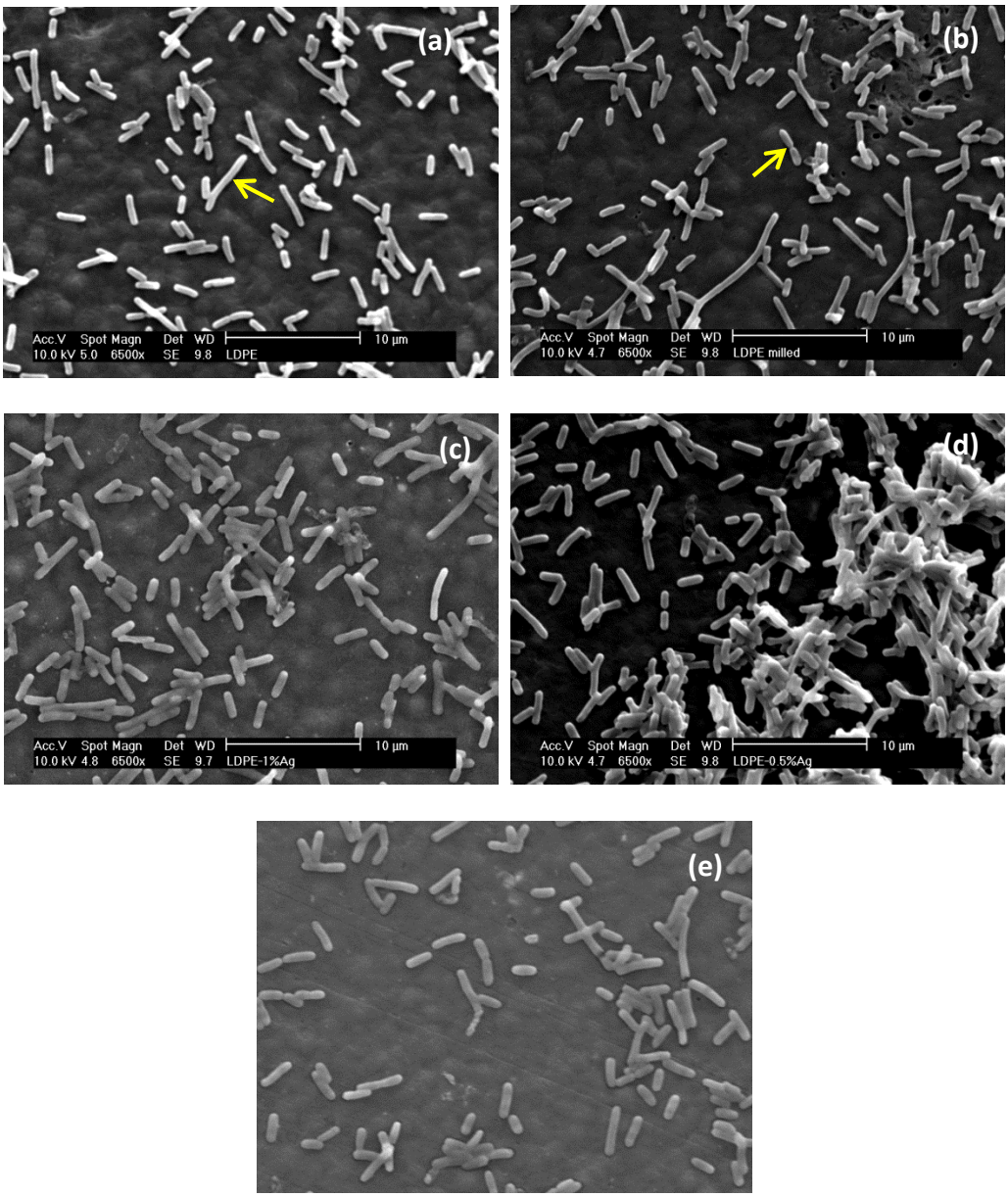

Figure 18. SEM micrographs with 6,500x magnification of bacterial cultures on (a) grinded PE, (b) milled PE, (c) PE + 0.5% Ag, (d) PE + 1% Ag and (e) PE + 2% Ag.

6.3.3. Morphological characteristics of bacteria

SEM images obtained at higher magnification (6,500x) corresponding to the surfaces of the materials after bacterial cultures (i.e., images shown in Figure 18) were analyzed using ImageJ software since are the ones with a better contrast between the materials and bacteria. Several studies were done in order to study the effect of silver concentration on bacterial morphology:

study of the mean width, length and aspect ratio of bacteria cultured on the surface of different films.

First of all, we calculate the width and length of 25 bacteria in each 6,500x magnification image. In order to get the distance in µm, we have to select in the program the right scale as follows:

1) Since the images have the scale bar, measure the distance of the scale bar (click on  , draw a line covering the scalebar, *Analyze*, *Measure*)

2) *Analyze*, *Set scale*

- Known distance= distance in µm of the scale bar (in this case 10 µm)

- Unit of length= um (micrometers)

After determining the scale, we measure the length and width of 25 different bacteria for each image on Figure 17 using again *Analyze*, *Measure*.

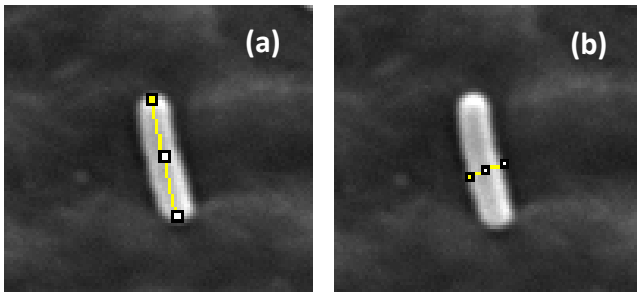

Figure 19. Examples of the measurements of (a) lentgh and (b) width of a bacterium.

Once all the measurements are performed, the mean bacterial width and length on each material can be calculated, as well as the mean aspect ratio (defined in terms of the ratio length/width). Results are presented in Figure 20 (mean width), Figure 21 (mean length) and Figure 22 (mean aspect ratio). For Figures 20 and 21, 0 Ag concentration (wt%) corresponds to both grinded and milled PE; 0.5 Ag concentration (wt%) corresponds to PE + 0.5% Ag; 1 Ag concentration (wt%) corresponds to PE + 1% Ag; and 2 Ag concentration (wt%) corresponds to PE + 2% Ag.

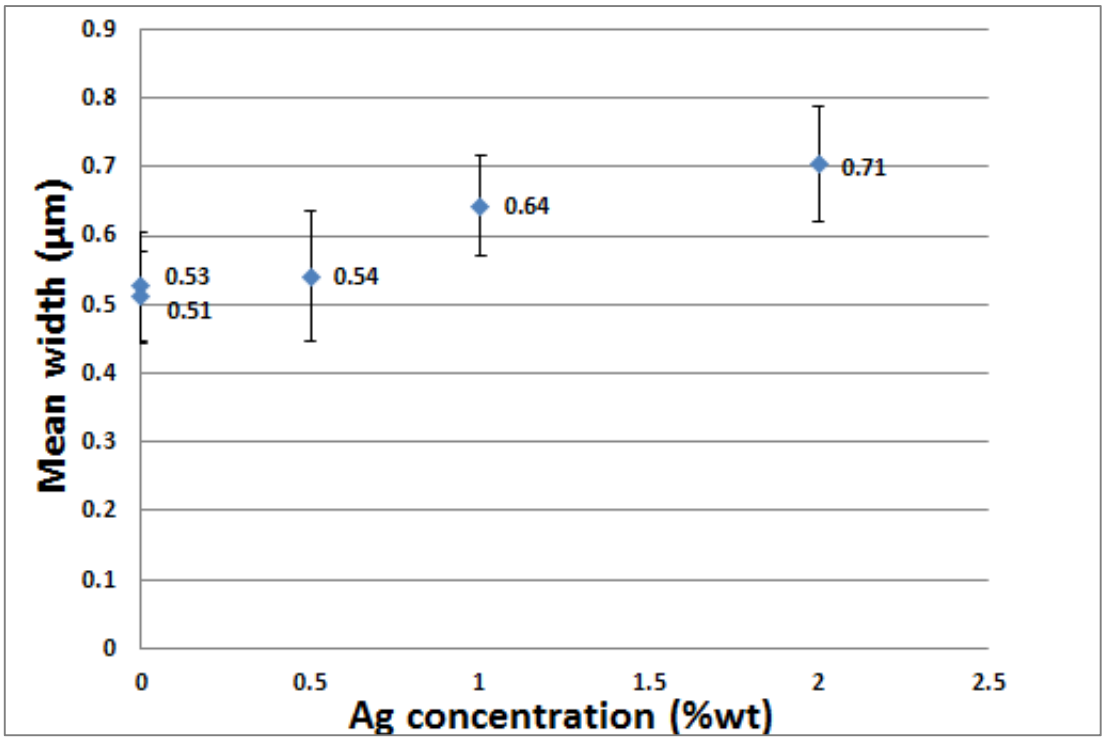

Figure 20. Mean bacterial width (in µm) for the materials under study as a function of Ag content (in %wt).

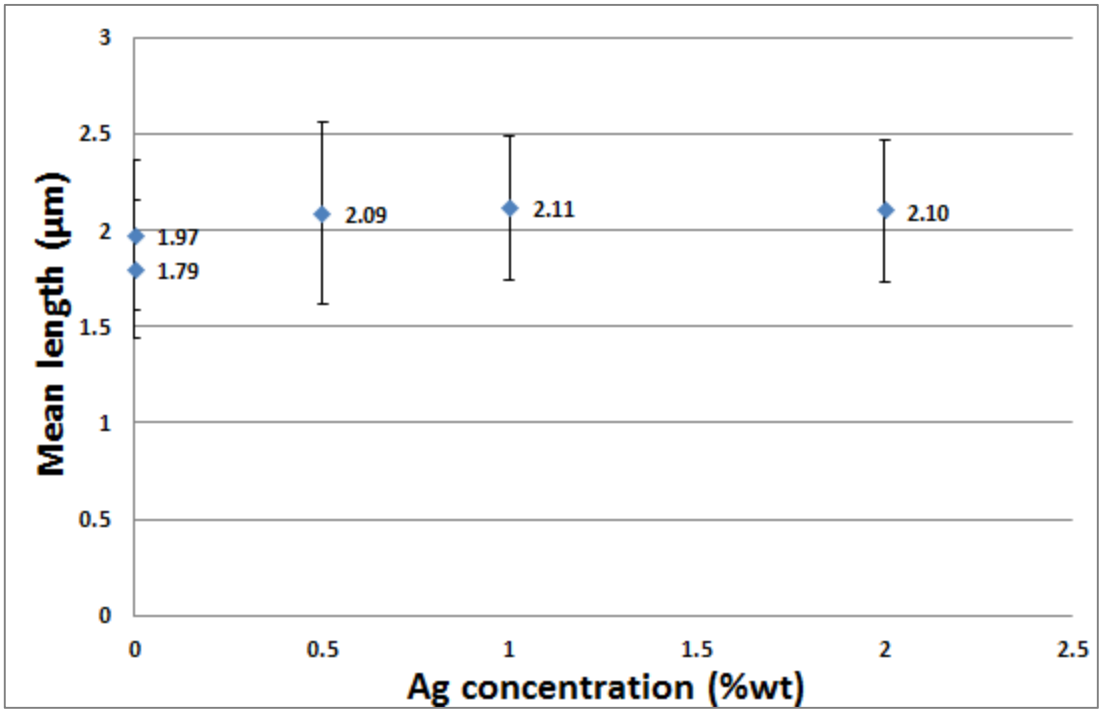

Figure 21. Mean bacterial length (in µm) for the materials under study as a function of Ag content (in %wt).

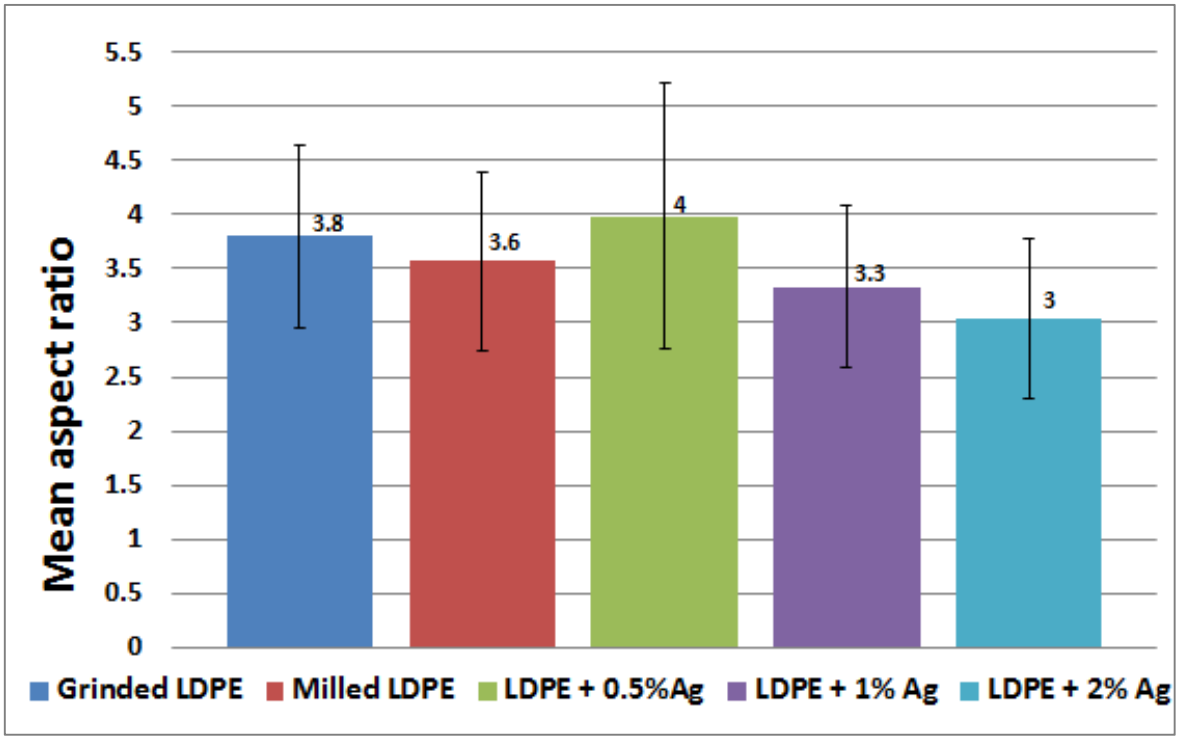

Figure 22. Mean aspect ratio of the bacteria on the surface of the materials under study.

In Figure 20, the variation of the mean width of bacteria with silver content is represented. It can be observed that the width increases smoothly as the concentration of Ag increases in the materials. More specifically, the width varies from 0.5 μm (for pure PE, either grinded or milled) to 0.7 μm for the sample with 2% AgNPs. In Figure 21, the mean length of bacteria with silver content is presented. It can be observed that the mean length slightly increases from materials without Ag to the PE + 0.5% Ag materials (1.9-2 μm) but it is constant in the three materials with different concentration of AgNPs (2.1 μm). Finally, regarding the aspect ratio (Figure 22) a slight decrease is observed as Ag concentration increases. This result is reasonable since width is increased while length remains more or less constant for almost all the materials.

Normally, an *E. coli* bacterium measures approximately 0.5 μm in width by 2 μm in length. These dimensions are preserved in grinded and milled PE films. On the other hand, width is increased 8.2%, 28.6% and 41% (PE + 0.5%, 1% and 2% Ag respectively). This significant change in width has led us to conclude that the increasing concentration AgNPs affect bacteria by increasing is width but not its length. Morphological changes observed in bacteria might be caused by the presence of AgNPs themselves. However, these changes are not due to changes in surface properties of the materials, as no significant variations in contact angle or surface energy were observed due to the presence of the particles. Therefore, there should be other possible reasons that cause these changes in bacteria.

As presented by Prabhu & Poulose, 2012 AgNPs have the ability to anchor to the bacterial cell wall and subsequently penetrate it, causing structural changes in the cell membrane. One possible explanation might be to consider that AgNPs may penetrate the cell wall and may, therefore, have caused a change in the osmolarity of bacteria causing an increase in its diameter by introducing themselves onto the microorganisms. This might be one plausible explanation for the materials if we consider that, as we increase the concentration of AgNPs in the material, the probability of finding some silver particles on the surface of the film increases, and so the ability of AgNPs to interact with the bacterial cell wall increases too.

6.4. Future work

In the project, five different samples were used, three of them with AgNPs in different concentrations. Further research should be done using higher concentrations of nanoparticles as for example, 5%, 10% and 20% wt. In this way, more drastic changes between materials with and without nanoparticles would be visible and would allow drawing clearer conclusions.

An interesting approach would be trying to increase the hydrophilicity of the surfaces. In the project, no changes in surface properties were produced when introducing silver to the materials. Therefore, these properties should be studied in detail to achieve an increase in hydrophilicity if possible.

More culture assays would be required to have clearly significant and valid results. Moreover, using other bacterial strain would be a good idea to study the effect of the materials on different types of biofilms. Finally, deeper microbiology studies related to morphological changes in bacteria due to the presence of the nanoparticles would be useful. In this way, we could know how AgNPs affect bacteria and exploit it to increase its antibacterial effect.

7. Conclusions

In this work, composites based on LDPE and AgNPs were prepared using HEBM followed by a subsequent hot pressing step to obtain films of the materials. Although the materials were fairly homogeneous, some gradients in the concentration of silver particles were visible, especially in the PE + 0.5% Ag sample. This may be due to the fact that a full dispersion of the aggregates was not attained.

SEM visualization of the samples revealed the presence of silver rich domains of approximately 400-500 nm in size that were already present in the commercial nanoparticles. This suggests that the process of HEBM was not able to separate these aggregates into individual particles. Other possible explanation for the formation of these aggregates may be promoted also by the re-press process during film preparation.

The presence of the silver particles did not modify surface properties of the materials. Contact angle measurements showed that increasing nanoparticles concentrations in the films did not affect hydrophobicity and surface free energies. Kirby-Bauer diffusion test revealed that no diffusion of silver occurred in the materials since the inhibition zone did not change when particles were present in the films. However, a reduced density of bacteria on the surface of the materials was visualized during SEM analysis of the bacterial cultures. This led us to conclude that the effect of the particles was carried out onto the surface of the films instead of occurring because of diffusion of the particles.

SEM image analysis displayed some changes in bacterial morphology as concentration of silver increased in the nanocomposites. Bacterial length was preserved in the five films whereas width was proportionally increased as silver concentration increased. These significant changes in width produced a decrease in the aspect ratio of bacteria that were visibly wider in the PE + 2% Ag composites.

As a result, we can conclude that studies on biofilm development on the surface of materials revealed that the introduction of AgNPs is effective against bacterial growth. However, bacterial growth is inhibited only in the surface of the materials. Changes in the aspect ratio of the bacteria were detected, though the mechanism of action of the particles in the materials is not clearly known yet, since no significant changes in surface properties of the materials were detected. Therefore, further studies to clarify the mechanism of action of silver in bacteria will be needed.

These facts open new insights into the use of AgNPs as additives in polymeric matrix working as antibacterial agents, as their use seems to reduce density of bacteria onto their surface when increasing Ag wt%.

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9. List of tables and figures

9.1 List of figures

9.2 List of tables